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(54) Title: RECOMBINANT ENDOTOXIN-NEUTRALIZING PROTEINS

(57) Abstract

In general, the invention features a recombinant endotoxin-neutralizing polypeptide (RENP) characterized by (i) an amino acid sequence, (ii) an amino acid sequence and structure that facilitates selective and specific binding to lipopolysaccharide and (iii) once bound to the lipopolysaccharide, provides endotoxin-neutralizing activity. Preferably, the RENP is composed of an amino acid sequence similar to, but not identical to, an amino acid sequence of BPI, LBP, or both. preferably, the RENP contains an LPS-binding domain derived from the amino acid sequence of BPI, LBP or both. Preferably, the RENPs are covalently bound to a molecule which enhances the half-life of the polypeptide. The RENPs of the invention can be used in pharmaceutical compositions for therapeutic and prophylactic regimens, as well as in various *in vitro* and *in vivo* diagnostic methods.

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RECOMBINANT ENDOTOXIN-NEUTRALIZING PROTEINS

Field of the Invention

This invention relates generally to the field of recombinant, endotoxin-neutralizing proteins, particularly to recombinant proteins which bind endotoxin and block endotoxin-mediated activation of biological systems.

Background of the Invention

Gram-negative infections are a major cause of morbidity and mortality, especially in hospitalized and immunocompromised patients. [Duma, *Am. J. of Med.*, 78 (Suppl. 6A):154-164 (1985); and Kreger et al., *Am. J. Med.*, 68:344-355 (1980)]. Although available antibiotics are generally effective in inhibiting growth of Gram-negative bacteria, they do not neutralize the pathophysiological effects associated with endotoxins. Endotoxin is a heat stable bacterial toxin composed of lipopolysaccharides (LPS) released from the outer membrane of Gram-negative bacteria upon lysis [Shenep et al., *J. Infect. Dis.*, 150(3):380-388 (1984)], and is a potent stimulator of the inflammatory response. Endotoxemia occurs when endotoxin enters the bloodstream resulting in a dramatic systemic inflammatory response.

Many detrimental *in vivo* effects of LPS result from soluble mediators released by inflammatory cells. [Morrison et al., *Am. J. Pathol.*, 93(2):527-617 (1978)]. Monocytes and neutrophils, which ingest and kill microorganisms, play a key role in this process. Monocytes and neutrophils respond to endotoxin *in vivo* by releasing solubl proteins with microbicidal,

proteolytic, opsonic, pyrogenic, complement-activating and tissue-damaging effects. These factors mediate many of the pathophysiological effects of endotoxin. For example, tumor necrosis factor (TNF), a cytokine released by endotoxin-stimulated monocytes, causes fever, shock, and alterations in glucose metabolism and is a potent stimulator of neutrophils. Other cytokines such as IL-1, IL-6, and IL-8 also mediate many of the pathophysiologic effects of LPS, as well as other pathways involving endothelial cell activation by tissue factor, kininogen, nitric oxide and complement.

Endotoxin-associated disorders result from extra-gastrointestinal exposure to LPS, e.g. administration of LPS-contaminated fluids, or Gram-negative infections. Endotoxin-associated disorders can also result when the natural epithelial barrier is injured and the normal Gram-negative flora breach this barrier. For example, endotoxin-associated disorders can occur (a) when there is ischemia of the gastrointestinal tract (e.g., following hemorrhagic shock or during certain surgical procedures), or (b) when systemic or local inflammation causes increased permeability of the gut to endotoxin or Gram-negative organisms. The presence of endotoxin and the resulting inflammatory response may result, for example, in endotoxemia, systemic inflammatory response syndrome (SIRS), sepsis syndrome, septic shock, disseminated intravascular coagulation (DIC), adult respiratory distress syndrome (ARDS), cardiac dysfunction, organ failure, liver failure (hepatobiliary dysfunction), brain failure (CNS dysfunction), renal failure, multi-organ failure and shock.

Examples of diseases which can be associated with Gram-negative bacterial infections or endotoxemia include bacterial meningitis, neonatal sepsis, cystic fibrosis,

inflammatory bowel diseas and liver cirrhosis, Gram-negative pneumonia, Gram-negative abdominal abscess, hemorrhagic shock and disseminated intravascular coagulation. Subjects who are leukopenic or neutropenic, including subjects treated with chemotherapy or immunocompromised subjects (for example with AIDS), are particularly susceptible to bacterial infection and the subsequent effects of endotoxin.

Several therapeutic compounds have been developed to inhibit the toxic effects of endotoxin, including antibacterial LPS-binding agents and anti-LPS antibodies, although each has met with limitations. For example, Polymyxin B (PMB) is a basic polypeptide antibiotic which binds to Lipid A, the most toxic and biologically active component of endotoxin. PMB inhibits endotoxin-mediated activation of neutrophil granule release *in vitro* and is a potential therapeutic agent for Gram-negative infections. However, because of its systemic toxicity, this antibiotic has limited therapeutic use, and is generally used topically. Combination therapy using antibiotics and high doses of methylprednisolone sodium succinate (MPSS) showed more promise as this regimen prevented death in an experimental animal model of Gram-negative sepsis. However, a clinical study using MPSS with antibiotics in treatment of patients having clinical signs of systemic sepsis showed that mortality rates were not significantly different between the treatment and placebo groups [Bone et al., *N. Engl. J. Med.* **317**:653 (1987)].

Antibodies that bind endotoxin have been used in the treatment of endotoxemia. For example, hyperimmune human antisera against *E. coli* J5 reduced mortality by 50% in patients with Gram-negative bacteremia and shock [Ziegler et al., *N. Engl. J. Med.* **307**:1225 (1982)]. However, attempts to treat Gram-negative sepsis by

administration of anti-LPS mon clonal antibodies met with little or no success [Ziegler et al., *N. Engl. J. Med.* **324**:429 (1991); Greenman et al., *JAMA* **266**:1097 (1991); Baumgartner et al., *N. Engl. J. Med.* **325**:279 (1991)].

Another approach to treating endotoxemia involves the use of cytokine blockers, such as IL-1 receptor antagonists and anti-TNF antibodies, as well as the soluble forms of the IL-1 and TNF receptors. However, any given cytokine blocker blocks only the cytokine for which it is specific, and fails to prevent the action of other cytokines. Furthermore, blocking cytokines may have other deleterious effects.

Two soluble endotoxin-binding proteins, lipopolysaccharide binding protein (LBP) and bactericidal/permeability-increasing (BPI), play opposing roles *in vivo* in the physiological response to endotoxin. LBP is a soluble LPS receptor found in serum which binds LPS with high affinity via interaction with the Lipid A moiety [Tobias et al. (1986) J. Exp. Med. 164:777-793; Tobias et al. (1989) J. Biol. Chem. 264:10867-10871]. LBP-LPS complexes stimulate monocyte activation through interaction with the CD14 receptor on the surface of monocytes, resulting in production of cytokines such as TNF and IL-1 [Wright et al. (1989) J. Exp. Med. 170:1231-1241; Wright et al. (1990) Science 249:1431]. Thus, LBP acts as a transfer protein in LPS-mediated stimulation of cytokine release. Moreover, LBP increases LPS activity in that a lower concentration of LPS is required to stimulate monocytes in the presence of LBP than in its absence.

In direct contrast to LBP, BPI binds and neutralizes endotoxin, preventing inflammatory cell activation. BPI, also known as CAP57 and BP [Shafer et al., *Infect. Immun.* **45**:29 (1984); Hovde et al., *Infect. Immun.* **54**:142 (1986)] is also

bactericidal by virtue of its interaction with the Lipid A moiety of LPS in the bacterial cell wall. BPI binds LPS, disrupts LPS structure and the cell wall, and increases bacterial membrane permeability, resulting in cell death [Weiss et al., J. Biol. Chem, 253:2664-2672 (1978); Weiss et al., Infection and Immunity 38:1149-1153 (1982)]. BPI retains its *in vitro* bactericidal activity after protease cleavage, suggesting that BPI fragments retain activity [Ooi et al., Clinical Research 33(2):567A (1985)]. This observation was confirmed by Ooi et al., who showed that an N-terminal 25 kD fragment of BPI exhibited both the *in vitro* bactericidal and permeability increasing activities [Ooi et al., J. Biol. Chem. 262:14891 (1987)].

Molecular Structures of BPI and LBP

The genes encoding BPI and LBP have been cloned [Gray et al. (1989) J. Biol. Chem. 264:9505-9509; Schumann et al., *Science* 249:1429-1431 (1990)]. BPI and LBP are immunologically cross-reactive, contain a hydrophobic leader sequence, and share significant amino acid sequence homology over the entire length of the molecules, with an overall amino acid sequence identity of 44% [Tobias et al., J. Biol. Chem. 263:13479-13481 (1988); Schumann et al. *supra*]. BPI and LBP each contains three cysteine residues. BPI contains two glycosylation sites; LBP contains five potential glycosylation sites.

BPI is characterized by two distinct domains, an N-terminal domain and a C-terminal domain, which are separated by a proline-rich hinge region. The N-terminal domain of BPI has strong LPS-neutralizing activity, while the C-terminal domain of BPI has modest LPS-neutralizing activity. LBP can also be divided into N- and C-terminal domains, with the C-terminal domain being implicated in

binding of LPS to macrophages and their subsequent activation.

The N- and C-terminal domains of BPI have a striking charge asymmetry that is not shared by LBP. The N-terminal domain of BPI, which is rich in positively charged lysine residues, imparts a predicted pI > 10 to the full-length molecule. In contrast, the C-terminal domain of BPI is only slightly negatively charged. LBP, which is a neutral protein, has no bactericidal activity [Tobias et al., *J. Biol. Chem.* **263**:13479 (1988)]. This suggests that the bactericidal activity of BPI results from its overall cationicity.

Table 1 provides a comparison of BPI and LBP structure and function.

Table 1

Comparison of BPI and LBP Structure and Function

	BPI	LBP
SYNTHESIS		
Site of synthesis	Neutrophil	Liver
Blood concentration	1-10 ng/ml	1-10 μg/ml
STRUCTURE		
Molecular mass	55 kD	60 kD
Glycosylation sites	2	5
Cysteine	3	3
EFFECTS ON LPS MEDIATED:		
neutrophil activation	Inhibits	Stimulates
monocyte activation	Inhibits	Stimulates
TNF release	Inhibits	Stimulates
IL-1 release	Inhibits	Stimulates
IL-6 release	Inhibits	Stimulates

* Four cysteines were reported by Schumann et al. [*Science* **249**:1429-1431 (1990)]. Subsequent DNA sequence analysis by the present inventors determined that Schumann's sequence was erroneous and that LBP contains only three cysteine residues (see Figure 1).

Therapeutic intervention to block the inflammatory effects of LPS would ameliorate the morbidity and mortality associated with endotoxemia and septic shock. Unfortunately, although BPI binds LPS with high affinity, it has an extremely short half-life *in vivo*, thus limiting its use in therapy. Native LBP has a longer half-life but, upon binding of LPS, elicits a brisk monocyte reaction which can facilitate release of deleterious quantities of cytokines.

Early and specific diagnosis of endotoxin-associated disorders is essential in the identification of patients who have or who are at risk of developing such disorders.

Precise identification of a site of Gram-negative infection in a patient would assist the clinician in the design and targeting of antibacterial therapy.

An ideal anti-endotoxin drug candidate and/or LPS detection reagent would have a longer half-life and effective, high-affinity endotoxin binding/inactivation without monocyte stimulation. There is a clear need in the field for specific diagnostic and therapeutic agents which neutralizes the effects of endotoxin and has an acceptably long half-life *in vivo*. The present invention addresses these problems.

Summary of the Invention

Recombinant proteins are genetically engineered to bind lipopolysaccharide (LPS) such that the endotoxin is inactivated, thus preventing the endotoxin from inducing the immunological cascade of events associated with endotoxin-related disorders (e.g., activation of monocytes, tumor necrosis factor (TNF) production).

In general, the invention features a recombinant endotoxin-neutralizing polypeptide (RENP) characterized by (i) an amino acid sequence, (ii) a sequence and structure that facilitate specific binding to lipopolysaccharide, (iii) provides endotoxin-neutralizing activity upon LPS binding, and (iv) a half-life that is enhanced relative to the half-life of BPI. Preferably, the RENP is composed of an amino acid sequence similar to, but not identical to, an amino acid sequence of BPI, LBP, or both. Preferably, the RENP contains an LPS-binding domain derived from the amino acid sequence of BPI, LBP, or both. Pref rred RENPs are fusion

proteins which bind LPS with the high affinity of BPI, but do not contain the BPI amino acid sequences associated with BPI's short half-life.

Preferably, the RENPs are covalently bound to a molecule which further enhances the half-life of the polypeptide. For example, the half-life enhancing molecule can be an immunoglobulin fragment, a half-life determining portion of LBP or LBP derivative, or polyethylene glycol. In related aspects, the invention features DNA encoding an RENP of the invention, vectors and transformed cells containing DNA encoding an RENP, a method for producing RENPs, and detectably labeled RENPs.

A primary object of the invention is to provide an RENP which binds and inactivates endotoxin, and has a half-life suitable for administration to a patient.

Another object of the invention is to provide a pharmaceutical composition containing a therapeutically effective amount of an RENP for use in treatment of endotoxin-related disorders.

Still another object of the invention is to provide endotoxin-neutralizing proteins for use in the detection of LPS. The RENPs can be bound to a label which can be detected or can be bound to a support for use in LPS-detection assays. LPS can be detected *in vivo* to identify a site of infection in a subject or can be used in an *in vitro* assay to qualitatively or quantitatively detect LPS in a sample.

Another object of the invention is to provide endotoxin-neutralizing proteins that can be used to produce endotoxin-free solutions and tools for use in, for example, various medical applications.

An advantage of the present invention is that the endotoxin-neutralizing proteins have a half-life in serum which is enhanced relative to the half-life of naturally-occurring LPS-binding proteins, and bind LPS

without triggering a significant, undesirable immune response.

Another advantage of the invention is that the RENPs can be administered to a patient immediately upon identification of a symptom of an endotoxin-associated disorder.

Another advantage is that the endotoxin-neutralizing proteins can be administered prophylactically to a patient at risk of endotoxic shock or other LPS-mediated condition.

An advantage of the invention is that various RENPs having binding specificity for LPS for detection of LPS either *in vivo* or *in vitro*.

Another advantage of the invention is that the RENPs can be attached to a variety of detectable labels.

Yet another advantage of the invention is that the RENPs can be bound to a molecule which can interact with or which can be a portion of a solid support.

These and other objects, advantages and features of the present invention will become apparent to those persons skilled in the art upon reading the details of the vectors, cell lines and methodology as more fully set forth below.

Brief Description of the Drawings

Figures 1A-1D are a comparison of the amino acid sequences of human LBP as described by Schumann et al. (LBPa) and as used herein (LBPb).

Figure 2 is a schematic diagram showing the various combinations of BPI, LBP, BPI variants, and/or LBP variants which can be used to generate an RENPs of the invention.

Figures 3A-3D show the nucleotide and amino acid sequences of BPI.

Figures 4A-4C show the nucleotide and amino acid sequences of LBP.

Figures 5A-5F are a comparison of the amino acid sequences of BPI and LBP from various species.

Figure 6 shows the amino acid sequence of $L_{1-197}B_{200-456}$ (NCY118).

Figure 7 is a graph showing the effects of BPI, LBP, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103) and $B_{1-199}L_{200-456}$ (NCY104) on ^{biotinylated}BPI binding to LPS.

Figure 8 is graph showing the effects of BPI, LBP, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103), $B_{1-199}L_{200-456}$ (NCY104), or $B_{(S351->A)}$ (NCY105) protein on LPS activity in the chromogenic LAL assay.

Figure 9 is a graph showing FITC-LPS binding to monocytes in the presence of BPI or $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103).

Figure 10 is a graph showing the effects of BPI, LBP, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103) or $B_{1-199}L_{200-456}$ (NCY104), on TNF release by LPS in whole blood.

Figure 11 is a graph showing clearance of BPI, LBP, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103) or $B_{1-199}L_{200-456}$ (NCY104) from mouse serum after intravenous injection.

Figure 12 is a graph comparing the efficacy of BPI and $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103) in the protection to endotoxin challenge.

Figures 13A-13C are graphs showing the effects of BPI, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103), $L_{1-197}B_{200-456}$ (NCY118), $L_{1-198}B_{201-456}Fc$ (NCY144), $L_{1-59}B_{60-456}$ (NCY114), $L_{1-134}B_{135-456}$ (NCY115), $L_{1-359}B_{360-456}$ (NCY117), and B_{CAT9} (NCY139) on biotinylated BPI binding to LPS.

Figures 14A-14B are graphs showing the effects of BPI, LBP, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103) and $B_{1-199}L_{200-456}$ (NCY104) on FITC-labeled LPS binding to human peripheral blood monocytes in the presence of 10%

autolog us serum (14A) and in the absence f serum and presence of 0.5% human serum albumin (14B).

Figure 15 is a graph comparing the effects of LBP vs. $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103), $B_{1-199}L_{200-456}$ (NCY104), $L_{1-359}B_{360-456}$ (NCY117) and PLL (poly-L-lysine) on the stimulation of TNFα release by phorbol ester-induced THP-1 cells in the absence of serum.

Figure 16 is a graph showing the effects of various recombinant-endotoxin neutralizing proteins upon LPS-mediated TNF production in THP-1 cells cultured without serum.

Figures 17A-17H are graphs showing the clearance of: BPI, LBP, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103), $B_{1-199}L_{200-456}$ (NCY104), and $L_{1-197}B_{200-456}$ (NCY118) (17A); BPI, $L_{1-59}B_{60-456}$ (NCY114), $L_{1-134}B_{135-456}$ (NCY115), and B_{CAT9} (NCY139) (17B); BPI, LBP, $L_{1-359}B_{360-456}$ (NCY117) and $L_{1-197}B_{200-456}$ (NCY118) (17C); and BPI, LBP and $L_{(1-198)}B_{(201-456)}Fc$ (NCY144) (assayed for both Fc and BPI) in CD-1 mice (17D); LBP, $L_{1-275}B_{278-456}$ (NCY116), $L_{1-359}B_{360-456}$ (NCY117), $L_{1-197}B_{200-456}$ (NCY118) (17E); LBP, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103), $L_{1-134}B_{135-456}$ (NCY115), $L_{(1-198)}B_{(202-275)}L_{(274-456)}$ (NCY135), and $L_{(1-134)}B_{(136-275)}L_{(274-456)}$ (NCY134) (17F); LBP (NCY102), L_{CAT6} (NCY141), L_{CAT9} (NCY142), L_{CAT15} (NCY143) and BPI (17G); and BPI, $L_{1-134}B_{135-456}$ (NCY115), and $L_{1-59}B_{60-456}$ (NCY114) (17H).

Figure 18 is Western blot of BPI and $L_{1-197}B_{200-456}$ (NCY118) produced in *Pichia pastoris*.

Figure 19 is a graph showing the effects of BPI and $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103) on endotoxin activation of monocytes.

Figure 20 is a graph showing the protective effects of $L_{1-197}B_{200-456}$ (NCY118) to endotoxin challenge in mice.

Description of the Preferred Embodiments

Before the present recombinant endotoxin-neutralizing proteins, methods for providing therapy to a patient suffering from an endotoxin-related disorder, and compositions and method for diagnosis of a condition associated with LPS are described, it is to be understood that this invention is not limited to the particular methodology, protocols, cell lines, vectors and reagents described as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

It must be noted that as used herein and in the appended claims, the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a recombinant endotoxin-neutralizing protein" includes a plurality of such proteins and reference to "the DNA encoding the recombinant endotoxin-neutralizing protein" includes reference to one or more transformation vectors and equivalents thereof known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, devices and materials are now described.

All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the cell lines, vectors, and methodologies

which are described in the publications which might be used in connection with the presently described invention.

Definitions

By "lipopolysaccharide" or "LPS" is meant a compound composed of a heteropolysaccharide (which contains somatic O antigen) covalently bound to a phospholipid moiety (lipid A). LPS is a major component of the cell wall of Gram-negative bacteria.

By "endotoxin" is meant a heat-stable toxin associated with the outer membranes of certain Gram-negative bacteria, including the enterobacteria, brucellae, neisseriae, and vibrios. Endotoxin, normally released upon disruption of the bacterial cells, is composed of lipopolysaccharide molecules (LPS) and any associated proteins. The phospholipid moiety of LPS, lipid A, is associated with LPS toxicity. When injected in large quantities endotoxin produces hemorrhagic shock and severe diarrhea; smaller amounts cause fever, altered resistance to bacterial infection, leukopenia followed by leukocytosis, and numerous other biologic effects. Endotoxin is a type of "bacterial pyrogen," which is any fever-raising bacterial product. The terms "endotoxin," "LPS," and "lipopolysaccharide" as used herein are essentially synonymous.

By "recombinant endotoxin-neutralizing polypeptide", "RENP" or "recombinant LPS-neutralizing polypeptide" is meant a protein which has been genetically engineered and contains an LPS-binding domain. Preferably, such recombinant LPS-binding proteins bind endotoxin, have a relatively long half-life in serum (e.g., compared to bactericidal/permeability increasing (BPI) protein), and elicit no or relatively little of the undesirable inflammatory side effects

associated with endotoxin and/ r binding of LPS to particular naturally occurring endotoxin-binding proteins (e.g., lipopolysaccharide binding (LBP) protein). "RENPs" of the invention do not occur naturally and are distinct from those endotoxin-binding proteins that do occur in nature, specifically BPI and LBP.

By "LPS-binding domain" is meant an amino acid sequence which confers specific and selective LPS binding upon a polypeptide.

By "high affinity LPS binding" is meant an LPS binding affinity greater than the LPS binding affinity of LBP, preferably about the same or greater than the LPS binding affinity of BPI.

By "endotoxin-neutralizing activity" is meant a biological activity associated with inhibition of the toxic effects of lipopolysaccharide, e.g., by binding LPS and preventing interaction of LPS with proteins and/or receptors which mediate an undesirable immunological response associated with endotoxin in a mammalian host.

By "recombinant" or "genetically engineered" is meant a DNA sequence, or a polypeptide encoded thereby, generated using nucleic acid manipulation techniques (e.g., cloning, PCR, and/or fusion protein techniques). "Recombinant" or "genetically engineered" DNA, and thus the proteins encoded by such DNAs, do not occur in nature.

By "half-life" is meant the time required for a living tissue, organ, or organism to eliminate one-half of a substance introduced into it.

By "molecule which enhances the half-life" or "half-life enhancing molecule" is meant chemical moiety (e.g., bound via a chemical modification) which enhances the biological half-life of a polypeptide with which it is associated relative to the biological half-life of the parent polypeptide. Chemical moieties include an amino

acid sequ nce or protein. For example, where a polyethylene glycol (PEG) moiety is covalently bound to a protein so as to increase the half-life of the protein relative to the un-PEGylated parent protein, the PEG moiety is the "molecule which enhances the half-life" of the protein.

By "half-life determining portion" of a polypeptide is meant an amino acid sequence which is associated with the biological half-life of the polypeptide.

By "bactericidal/permeability increasing protein" or "BPI" is meant a naturally occurring or recombinantly expressed protein having the DNA and amino acid sequences shown in Figures 3A-3D.

By "lipopolysaccharide binding protein" or "LBP" is meant a naturally occurring or recombinantly expressed protein having the DNA and amino acid sequences shown in Figures 1A-1D and Figures 4A-4C.

By "BPI variant" is meant a protein having an amino acid sequence similar to, but not identical to, the amino acid sequence of BPI. "BPI variants" (a) bind LPS, (b) competitively bind LPS in the presence of BPI or LBP, and (c) inhibit the LPS-mediated production of TNFα by human monocytes. In general, "BPI variants" contain the amino acid sequence of BPI but with at least one of: 1) an amino acid substitution; 2) an amino acid deletion; or 3) an amino acid addition, relative to the BPI amino acid sequence.

By "LBP variant" is meant a protein having an amino acid sequence similar to, but not identical to, the amino acid sequence of LBP. "LBP variants" (a) bind LPS, (b) competitively bind LPS in the presence of BPI or LBP, and (c) inhibits production of TNFα by human monocytes. In general, "LBP variants" contain the amino acid sequence f LBP but with at least one of: 1) an amino

acid substitution; 2) an amino acid deleti n; or 3) an amino acid addition, relative to the LPB amino acid sequence.

By "detectable label" is meant any molecule recognized in the art as a means for identifying and/or detecting a protein to which the detectable label is bound. Exemplary "detectable labels" include radionucleotides, fluorescent moieties, biotin, and antigenic molecules (e.g., a polypeptide which is specifically bound by an anti-polypeptide antibody). "Detectable labels" include a portion of a chimeric protein where a portion of the chimeric protein can be detected by, for example, binding of a detectably labeled antibody or other detectably labeled molecule which specifically binds the chimeric protein portion.

By "support" is meant a surface to which LPS or an RENP of the invention can be bound and immobilized. Exemplary supports include various biological polymers and non-biological polymers.

By "condition associated with endotoxin", "endotoxin associated disorder", or "endotoxin-related disorder" is meant any condition associated with extra-gastrointestinal (e.g., mucosal, blood-borne, closed space) lipopolysaccharide, e.g., a condition associated with bacteremia or introduction of lipopolysaccharide into the blood stream or onto an extra-gastrointestinal mucosal surface (e.g., the lung). Such disorders include, but are not limited to, endotoxin-related shock, endotoxin-related disseminated intravascular coagulation, endotoxin-related anemia, endotoxin-related thrombocytopenia, endotoxin-related adult respiratory distress syndrome, endotoxin-related renal failure, endotoxin-related liver disease or hepatitis, systemic immune response syndrome (SIRS) resulting fr m Gram-negative infection, Gram-negative

neonatal sepsis, Gram-n gative meningitis, Gram-negative pneumonia, neutropenia and/or leucopenia resulting from Gram-negative infection, hemodynamic shock and endotoxin-related pyresis.

By "transformation" is meant a permanent genetic change induced in a cell following incorporation of new DNA (i.e., DNA exogenous to the cell). Where the cell is a mammalian cell, the permanent genetic change is generally achieved by introduction of the DNA into the genome of the cell.

By "transformed cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding a protein of interest.

By "promoter" is meant a minimal DNA sequence sufficient to direct transcription. "Promoter" is also meant to encompass those promoter elements sufficient for promoter-dependent gene expression controllable for cell-type specific, tissue-specific or inducible by external signals or agents; such elements may be located in the 5′ or 3′ regions of the native gene.

By "operably linked" is meant that a DNA sequence and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s).

By "operatively inserted" is meant that the DNA of interest introduced into the cell is positioned adjacent a DNA sequence which directs transcription and translation of the introduced DNA (i.e., facilitates the production of, e.g., a polypeptide encoded by a DNA of interest).

By "mammalian subject" or "mammalian patient" is meant any mammal for which the therapy of the invention

is desired, including human, bovine, equine, canine, and feline subjects.

The invention will now be described in further detail.

Nomenclature used to describe RENPs

In order to facilitate the discussion and description of the RENPs of the invention, each RENP is designated a specific formula to briefly describe the amino acid sequence of the protein, as well as the origin of specific portions of the protein. The portion of BPI in the recombinant protein is designated with the letter B, followed by an amino acid sequence numbering assignment corresponding to that shown in Figures 5A-5F for human BPI, wherein the mature N-terminus is designated as residue 1. The portion of LBP in certain LBP variants and chimeras is designated by the letter L, followed by an amino acid sequence numbering assignment corresponding to that shown in Figures 1A-1D for human LBP, wherein the mature N-terminus is designated as residue 1. To avoid confusion between the erroneous LBP amino acid sequence published by Schumann et al., *supra* (designated LBP-a) and the correct LBP amino acid sequence used in the RENPs of the invention (designated LBP-b) and presented in Figures 1A-1D. The differences between the DNA and amino acid sequences for "LBP-a" and "LBP-b" are presented in Table 2A below.

As an example of RENP nomenclature, $L_{1-197}B_{200-456}$ (NCY118) contains amino acid residues 1-199 of LBP fused at the C-terminus of the LBP portion to the N-terminus of amino acid residues 200-456 of BPI. $L_{1-197}B_{200-456}$, shown in Figure 6 has the N-terminal domain of LBP (having a putative endotoxin-binding domain) fused to the C-terminal domain of BPI (having a putative LPS-clearing domain).

In this application, single amino acid residue substitutions are noted in parentheses, wherein the original amino acid residue is indicated (using the standard one letter code for amino acids), followed by the substitute amino acid residue. For example, the BPI variant having an alanine residue substituted for the original serine residue at position 351 (which substitution removes a glycosylation signal) is designated $BPI_{(S351->A)}$. In another example, in $B_{(DS200->DP)}$, a proline residue is substituted for the serine residue at position 200. In this latter example, the amino acid substitution produces a formic acid-cleavable site.

As another example, the RENP LBP-BPI chimera NCY103 is designated $L_{1-198(I43->V)}B_{201-456(D206->N)}$. In the recombinant protein, the original isoleucine residue at position 43 of the LBP portion is substituted with a valine residue, and the original asparagine residue at position 206 of the BPI portion is substituted with an aspartate residue. The C-terminus of the LBP amino acid sequence 1-198 having isoleucine substituted at position 43 is covalently bound to the N-terminus of the BPI amino acid sequence 201-456 having valine substituted at position 206.

The amino acid substitutions may be substitutions wherein an original amino acid residue at a given position is substituted with the residue at the corresponding position in a different protein. $BPI_{(Xn->Y)}$ is an example of such a substitution, wherein amino acid residue X at position n in BPI is substituted with residue Y which is found at position n in LBP (or rabbit or bovine LBP). "X" and "Y" denote amino acid positions in a primary amino acid sequence. "Y" as used in this context is not to be confused with the symbol "Y" denoting the amino acid residue tyrosine. $LBP_{(Xn->Y)}$ is

another example of such a substitution, wherein amino acid residue X at position n in LBP is substituted with residue Y which is found at position n in BPI (or rabbit or bovine BPI).

Amino acid residue insertion changes are noted in parentheses, by indicating the amino acid residue after which the insertion occurs, followed by the amino acid residue after which the insertion occurs together with the inserted residue or residues. For example, $B_{(D200papain)}$ indicates that an amino acid sequence for cleavage of the BPI variant by papain is inserted after the aspartic acid at residue position 200.

TABLE 2A

Individual Sequence Differences Between Schumann et al. and LBP as Used Herein

NUCLEIC ACID		PROTEIN	
Alpha	*Beta*	*Alpha*	*Beta*
A_{42}	C_{42}	$G_{129}YCL_{132}$	$V_{129}TAS_{132}$
C_{318}	T_{318}	S_{149}	F_{149}
G_{488}	(np)	A_{241}	$V_{241}MSLP_{245}$
(np)	C_{499}	L_{411}	F_{411}
T_{546}	C_{546}		
C_{548}	T_{548}		
(np)	$T_{824}CATGAGCCTTC_{835}$		
C_{1333}	T_{1333}		

(np) = not present in the sequence

Table 2B describes some exemplary general classes of RENPs of the invention. In the formulas in Table 2B, n represents an amino acid residue position in the mature sequence of BPI or LBP, x represents an amino acid residue in a position which is C-terminal to n in the

sequence of BPI or LBP, and y represents an amino acid residue in a position which is C-terminal to x in the sequence of BPI or LBP. The symbols n, x and y denote the amino acid residue positions as they occur in the mature sequence of the native protein, and not necessarily the positions as they occur in the variant protein.

Table 2B

Examples of RENPs

BPI variant (N-terminal frag.)	B_{1-n}
LBP variant (N-terminal frag.)	L_{1-n}
BPI variant (C-terminal frag.)	B_{n-456}
LBP variant (C-terminal frag.)	L_{n-456}
BPI variant (internal frag.)	B_{n-x}
LBP variant (internal frag.)	L_{n-x}
LBP-BPI chimera	$L_{n-x}B_{(x+1)-y}$
BPI-LBP chimera	$B_{n-x}L_{(x+1)-y}$
LBP-BPI chimera	$L_{n-x}B_{(x+1)-456}$
BPI-LBP chimera	$B_{n-x}L_{(x+1)-456}$
LBP-BPI chimera	$L_{1-n}B_{(n+1)-x}$
BPI-LBP chimera	$B_{1-n}L_{(n+1)-x}$
LBP-BPI chimera	$L_{1-n}B_{(n+1)-456}$
BPI-LBP chimera	$B_{1-n}L_{(n+1)-456}$
LBP-BPI-LBP chimera	$L_{1-n}B_{(n+1)-x}L_{(x+1)-456}$
BPI-LBP-BPI chimera	$B_{1-n}L_{(n+1)-x}B_{(x+1)-456}$

All of the constructs in Table 2B can also contain additional molecules which confer an enhanced half-life upon the RENP (e.g., the RENP can be covalently bound to a polyethylene glycol moiety, or a portion of an immunoglobulin protein or other amino acid sequence which confers a half-life increased relative to the unmodified protein). The general scheme for generation of RENPs is outlined in Figure 2.

Production of RENPs

The RENPs of the invention minimally have characteristics associated with (i) specific and high affinity binding to lipopolysaccharide and (ii) endotoxin-neutralizing activity. In general, the amino acid sequence of RENPs is based upon an amino acid sequence of BPI, LBP, or both. However, the amino acid sequences of the RENPs are distinct from that of BPI and LBP, i.e. the RENPs contain amino acid substitutions, deletions, and/or additions relative to the amino acid sequence of BPI or LBP. Thus, the RENPs of the invention contain: 1) amino acid sequences of a naturally-occurring LPS-binding protein (i.e., LBP and/or BPI); and/or 2) amino acid sequences which do not occur within a single naturally-occurring LPS-binding protein (i.e., LBP or BPI). RENPs can thus be similar to, but not identical to, LBP or BPI. For example, the RENPs can be fragments of BPI and/or LBP, as the amino acid sequences of such RENPs are similar to, but not identical to, naturally occuring BPI or LBP. Moreover, the RENPs of the invention generally have biological properties distinct from and advantageous to either BPI or LBP. RENPs of the invention include BPI variants, LBP variants, and chimeric proteins composed of amino acid sequences derived from BPI, LBP, BPI variants, and/or LBP variants.

For example, RENPs can contain an amino acid sequence of BPI, where the BPI amino acid sequence 1) has been altered at a site of glycosylation (e.g., insertion or deletion of a glycosylation site); 2) contains a neutral or anionic amino acid substituted at a cationic residue of the BPI amino acid sequence (cationic substitution variants); 3) contains an amino acid substitution at a position normally occupied by cysteine in the BPI sequence (cysteine substitution variants); 4)

contains an amino acid substitution where the substituted amino acid is the amino acid at the corresponding position in the LBP amino acid sequence; and/or 5) contains an insertion or deletion of one or more secondary structure-altering amino acid residues.

Exemplary BPI variants containing a glycosylation site alteration include BPI variants having an amino acid residue other than serine substituted for the serine residue at position 351 of the BPI amino acid sequence. BPI variants of this type are of the formula BPI(S351->X), wherein X is any amino acid other than serine. Preferably, the amino acid substituted at position 351 is alanine. Other BPI variants having a glycosylation site deleted can be generated by, for example, other amino acid substitutions within the glycosylation site.

Additional exemplary BPI variants contain a neutral or anionic amino acid substituted at a cationic residue of the BPI amino acid sequence (cationic substitution variants). For example, one or more of the nonconserved positively-charged residues in BPI (i.e., those residues not found at the corresponding positions in LBP) can be substituted with the corresponding residue or residues in LBP, thus rendering BPI less cationic. Preferably, the cationic substitution variant contains an amino acid substitution in at least one of BPI amino acid residue positions 27, 30, 33, 42, 44, 48, 59, 77, 86, 90, 96, 118, 127, 148, 150, 160, 161, 167, 169, 177, 185, or 198. The cationic substitution variant can contain multiple amino acid substitutions. For example, the cationic substitution variant can contain a neutral or anionic residues at 1) BPI amino acid residue positions 27, 30, 33, 42, 44, 48, and 59; 2) BPI amino acid residue positions 77, 86, 90, 96, 118, and 127; 3) BPI amino acid residue positions 148, 150, 160, 161, 167, 169, 177, 185,

and 198; or 4) BPI amino acid residue positions 27, 30, 33, 42, 44, 48, 59, 77, 86, 90, 96, 118, 127, 148, 150, 160, 161, 167, 169, 177, 185, and 198.

Further example BPI variants contain an amino acid substitution at a position normally occupied by cysteine in the BPI sequence (cysteine mutant). The amino acid selected for substitution at this site can be the amino acid in the corresponding position in LBP. For example, a cysteine residue in BPI (which is not conserved in LBP) may be substituted with an alanine residue (the corresponding residue in LBP). Preferably, the amino acid substitution is at a cysteine residue at BPI amino acid residue position 132, 135, or 175. Preferably, alanine or serine is substituted for cysteine. More preferably, alanine is substituted for the cysteine at position 132 of BPI. Cysteine substitution mutants of BPI can prevent aggregation of the resulting RENPs during their production or use.

Another example of a BPI variant includes a BPI variant having an amino acid substitution where the substituted amino acid is the amino acid at the corresponding position in LBP. The amino acid at the corresponding position is determined by aligning the BPI and LBP amino acid sequences so as to maintain the highest level of amino acid sequence identity between the two sequences. For example, an RENP having the formula $B_{(Q329 \rightarrow S)}$ contains a substitution of the glutamine at BPI residue position 329 with the serine residue at the corresponding LBP residue position 327 (see Figures 5A-5F).

Additional exemplary BPI variants contain an insertion or deletion of one or more secondary structure-altering amino acid residues. For example, one or more of the nonconserved proline residues in BPI may

be substituted with the corresponding non-proline residue in LBP.

Alternatively, or in addition to the amino acid sequence of BPI and/or a BPI variant, the RENPs can contain an amino acid sequence of LBP, where the LBP amino acid sequence 1) has been altered at a site of glycosylation (e.g., insertion or deletion of a glycosylation site); 2) contains a cationic amino acid substituted at a neutral or anionic amino acid of the LBP amino acid sequence (cationic replacement mutant); 3) contains an amino acid substitution where the substituted amino acid is the amino acid at the corresponding position in the BPI amino acid sequence; and/or 4) contains an insertion or deletion of one or more secondary structure-altering amino acid residues. The LBP DNA and amino acid sequence used in the construction of particular RENPs exemplified herein is the amino acid sequence of human LBP in Figs. 5A-B.

Exemplary LBP variants contain a cationic amino acid substituted at a neutral or anionic amino acid of the LBP amino acid sequence (cationic replacement variant). For example, one or more of the nonconserved amino acid residues in LBP (at a position which corresponds to a positively-charged residue in BPI) may be substituted with the corresponding positively-charged residue in BPI, and thus result in an LBP variant having an increased positive charge, thus enhancing binding to the negatively charged phosphate groups in LPS, and/or facilitating interaction with the negatively charged surfaces of Gram-negative bacteria. Positively-charged residues include, by way of example, lysine, arginine, and histidine. Preferably, the substituted cationic amino acid is at least one of LBP amino acid residue positions 77, 86, 96, 118, 126, 147, 148, 158, 159, 161, 165, 167, 175, 183, or 196. Cationic replacement

variants can contain multiple amino acid residu substitutions at any combination of the amino acid residues recited above.

Other exemplary LBP variants include an LBP variant having an amino acid substitution where the substituted amino acid is the amino acid at the corresponding position in BPI. For example, $L_{(A401 \rightarrow D)}$ contains a substitution of the alanine residue of LBP at position 401 with the aspartic acid residue at the corresponding BPI residue position 403.

Further exemplary LBP variants contain an insertion or deletion of one or more one or more secondary structure-altering amino acid residues. For example, one or more of the nonconserved amino acid residues in LBP (at a position which corresponds to a proline in BPI) may be substituted with a proline residue. Preferably, such amino acid alterations alter the secondary structure of the resulting LBP variant so that it is more like the secondary structure of BPI.

Preferably, the RENPs of the invention contain at least one LPS-binding domain of BPI, LBP, a BPI variant, and/or a LBP variant. For example, the LPS-binding domain can be derived from BPI and/or LBP amino acid sequences 17-45, 65-99, and/or 141-167. Preferably, the RENP has an LPS binding affinity that is greater than the LPS binding affinity of LBP, more preferably an LPS binding affinity that is the same or greater than the LPS binding affinity of BPI. Preferably, the RENP has an LPS binding affinity that is about 25-fold to 50-fold, preferably about 50-fold to 100-fold, more preferably about 100-fold to 300-fold greater than the LPS binding affinity of LBP as determined by LPS binding or LPS binding competition assays. The LPS binding affinity of BPI is about 60-fold to 100-fold greater than the LPS binding affinity of LBP.

The RENPs can contain multipl LPS-binding domains derived from any of these LPS-binding proteins. For example, an RENP can be a multivalent chimeric protein (i.e., a fusion protein) composed of an LPS-binding domain of BPI covalently bound (i.e., as in a fusion protein) to an LPS-binding domain of LBP. As used herein, a chimera means a protein comprising all or a portion of a first protein fused to all or a portion of a second protein, which resulting fusion protein may in turn be fused to all or a portion of a third protein. Examples of chimeras include, by way of example, (a) a protein comprising a portion of LBP fused to a portion of BPI, (b) a protein comprising a portion of LBP fused to a portion of BPI which portion of BPI is in turn fused to a portion of an immunoglobulin protein, or (c) a protein comprising a portion of LBP fused to a portion of BPI, which is in turn fused to a portion of LBP. Each protein portion of the chimera may comprise a fragment of the protein, a point mutant of the protein (i.e., a variant), a deletion mutant of the protein, or a point and deletion mutant of the protein.

Examples of BPI fragments which can be incorporated into the RENPs of the invention include the BPI amino acid sequences 1-25, 1-85, 1-137, 1-135, 1-147, 1-159, 88-100, 148-161, 137-199, 44-159, 44-199, 135-199, 100-199, 162-199, 100-147. Examples of LBP fragments which can be incorporated into the RENPs of the invention include LBP amino acid sequences 1-43, 1-87, 26-135, 26-134, 86-99, 101-146, 101-197, 135-197, 137-197, 158-197, 160-197, and/or 147-159. The amino acid sequences of BPI and/or LBP can be comined in any order from N- to C-terminus to provide an RENP having sequences derived from BPI and/or LBP. For example, the RENPs can have the sequences $B_{1-137}L_{137-197}$, $L_{1-43}B_{44-199}$, $B_{1-159}L_{158-197}$, $B_{1-135}L_{135-197}$, $L_{1-43}B_{44-159}L_{158-197}$, $B_{1-25}L_{26-135}B_{137-199}$, $B_{1-25}L_{26-}$

$_{134}B_{135-199}$, $L_{1-87}B_{88-100}L_{101-146}B_{148-161}L_{160-197}$, $B_{1-85}L_{86-99}B_{100-199}$, $B_{1-147}L_{147-159}B_{162-199}$, $B_{1-85}L_{86-99}B_{100-147}L_{147-159}B_{162-199}$, $L_{1-87}B_{88-100}L_{101-197}$, or various combinations of other BPI and/or LBP fragments.

RENPs can share properties of both BPI and LBP. For example, fusing the N-terminal domain of LBP to the C-terminal domain of BPI results in an RENP which differs from LBP in that the chimera neutralizes endotoxin in whole blood and differs from BPI in that the chimera has a longer circulating half-life *in vivo*. Such RENPs have significant diagnostic and therapeutic potential. As per the nomenclature described above, RENPs designated BPI-LBP contain all or a part of the N-terminal domain of BPI fused to all or a part of the C-terminal domain of LBP. Likewise, RENPS designated LBP-BPI contain all or a part of the N-terminal domain of LBP fused to all or a part of the C-terminal domain of BPI.

Where the RENP contains amino acid sequences derived from both BPI and LBP, the RENP is preferably composed of a C-terminal fragment of BPI (or a BPI variant) and an N-terminal fragment of LBP (or an LBP variant). Preferably the C-terminal fragment of BPI (or a BPI variant) contains amino acid residues 60-456, 136-456, 199-456, 277-456, 300-456, 200-456, 136-361, 136-275, 200-275, or 200-361, more preferably 60-456, more preferably 199-359. The amino acid sequence of BPI from residue 199 to residue 359 contains a region required for neutralizing LPS, i.e., preventing LPS from stimulating an inflammatory response. Preferably, the N-terminal fragment of LBP (or an LBP variant) contains amino acid residues 1-59, 1-134, 1-164, 1-175, 1-274, 1-359, 1-134, or 1-197, more preferably 1-175. In addition to the specific amino acid sequences of BPI and LBP recited above, the RENP can also contain amino acid residues derived from the C-terminus of LBP (or an LBP

variant), preferably LBP (or LBP variant) amino acid residues 360-456 or 274-456.

Polypeptides which bind LPS can be identified using any of several assays well known in the art such as the 1) chromogenic LAL competition assay, 2) binding to LPS immobilized on a surface, and 3) FITC-LPS assay for binding to macrophages. The ability of a polypeptide to neutralize endotoxin can also be determined using methods well known in the art. Endotoxin neutralization assays include assays to examine the ability of a polypeptide to 1) prevent LPS-induced TNF release in whole blood, 2) inhibit or prevent TNF production by THP-1 cells, 3) provide protection in a mouse endotoxin challenge assay, and 4) reduce or prevent LPS-induced cytokine release and/or mortality in an animal model. Each of these assays are described in detail in the examples section below. The results of the *in vitro* and *in vivo* assays used herein are accepted in the art. The results of these assays are predictive of relevant biological activity *in vivo*, e.g. in humans.

Preferably, the RENPs of the invention have a biological half-life (e.g., serum half-life) which is enhanced relative to the biological half-life of BPI. Preferabl, the half-life of the RENP is enhanced relative to BPI such that the clearance time of the RENP is at least 1.5-fold to 10-fold, preferably about 10-fold to 50-fold, more preferably about 50-fold to 100-fold, even more preferably about 100-fold to 350-fold slower than the clearance rate of BPI. The clearance rate values representing these ranges are from about 8 ml/min to 1.5 ml/min, preferably 1.5 ml/min to 0.26 ml/min, more preferably 0.26 ml/min to 0.13 ml/min, even more preferably about 0.13 ml/min to 0.03 ml/min.

To enhance the RENP half-life, the RENP can be covalently bound to a molecule which enhances the

half-life of the polypeptide. The half-life enhancing molecule can be any of a variety of half-life enhancing molecules. Exemplary half-life enhancing molecules include immunoglobulin fragments, a half-life determining portion of LBP, a half-life determining portion of an LBP variant, or polyethylene glycol (PEG), preferably a half-life determining portion of LBP or an LBP variant. Preferably, where the half-life enhancing molecule is a portion of LBP or an LBP variant, the half-life enhancing molecule is derived from the N-terminus of the LBP or LBP variant amino acid sequence, more preferably from amino acid residues 1-59, 1-134, 1-274, 1-359, 1-134, 1-164, 1-175, or 1-197, most preferably 1-164 or 1-175. Methods of attachment of PEG moieties to a protein (i.e., PEGylation) are well known in the art and are exemplified in U.S. patent nos. 4,179,337; 5,166,322; 5,206,344; and PCT application serial no. PCT/US94/11624, published April 28, 1995.

As used herein, an RENP-Ig chimeric protein is an RENP which (i) contains a portion of BPI or LBP (at least 10 amino acid residues in length of (a) BPI, or (b) BPI variant, or (c) LBP, and/or (d) LBP variant) fused at the C-terminus to the N-terminus the Fc portion of an immunoglobulin molecule, and (ii) is capable of (a) binding to LPS, (b) competing with BPI or LBP for binding to LPS, and (c) inhibiting the production of TNFα by human monocytes. For example, the portion of the immunoglobulin molecule is derived from an IgG molecule, specifically from an IgG_1 heavy chain Fc domain. RENP-Ig chimera is a fusion protein composed predominantly of sequences derived from BPI, variant BPI, LBP and/or variant LBP. The term "LBP-BPI-IgG chimera" indicates that the RENP-Ig chimera contains amino acid sequences derived from both BPI (or a BPI variant) and LBP (or an LBP variant).

Identificati n of a half-life enhancing polypeptide sequence (e.g., a polypeptide derived from an immunoglobulin, LBP, or LBP variant) can be accomplished using methods well known in the art. For example, the test polypeptide with and without the half-life enhancing molecule bound to it are injected into an animal model to determine the effects of the putative half-life enhancing molecule. If the half-life of the polypeptide with the molecule is enhanced relative to the half-life of the polypeptide without the molecule, then the molecule is a half-life enhancing molecule suitable for use in the RENPs of the invention. For example, a putative half-life enhancing amino acid sequence is incorporated into a fusion protein with BPI. Both native BPI and the BPI fusion protein are injected into mice. If the BPI fusion protein has a half-life significantly greater than the half-life of native BPI, then the amino acid sequence in the BPI fusion has half-life enhancing characteristics, and thus can be incorporated into the RENPs of the invention.

Vectors and constructs

Any nucleic acid vector can be used to express DNA encoding an RENP of the invention. The vectors containing the DNA sequence (or the corresponding RNA sequence) which may be used in accordance with the invention may be any prokaryotic or eukaryotic expression vector containing the DNA (e.g., cDNA) or the RNA sequence of interest. A variety of suitable vectors are publicly available and well known in the art. For example, a plasmid can be cleaved to provide linear DNA having ligatable termini. These termini are bound to exogenous DNA having complementary, like ligatable termini to provide a biologically functional recombinant

DNA molecule having an intact replicon and a desired phenotypic property.

A variety of techniques are available for DNA recombination in which adjoining ends of separate DNA fragments are tailored to facilitate ligation. The vector is constructed using known techniques to obtain a transformed cell capable of expression of the RENP. The transformed cell is obtained by contacting a target cell with a RNA- or DNA-containing formulation permitting transfer and uptake of the RNA or DNA into the target cell. Such formulations include, for example, plasmids, viruses, liposomal formulations, or plasmids complexed with polycationic substances such as poly-L-lysine or DEAC-dextran, and targeting ligands. Transformed cells containing a construct encoding an RENP of the invention are also known in the art as "host vector systems". Vectors for use in the construction of constructs encoding the RENPs of the invention, as well as methods for molecular cloning, nucleic acid manipulation, and transformation of both eukaryotic and prokaryotic host cells are well known in the art (see, for example, Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd Ed., 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; hereby incorporated by reference with respect to bacterial and eukaryotic vectors, and methods and compositions for molecular cloning, nucleic acid manipulation, and transformation techniques).

The constructs of the invention may include promoter sequences to enhance expression of the RENP-encoding DNA, as well as other sequences (e.g., enhancers) which facilitate or enhance DNA expression. In addition, the RENP-encoding constructs can contain other components such as a marker (e.g., an antibiotic resistance gene (such as an ampicillin resistance gene) or β-galactosidase) to aid in selection of cells

containing and/or expressing the construct, an origin of replication for stable replication of the construct in a bacterial cell (preferably, a high copy number origin of replication), a nuclear localization signal, or other elements which facilitate production of the DNA construct, the protein encoded thereby, or both.

In general, the RENPs of the invention are constructed from a DNA sequence encoding BPI, a BPI variant, LBP, an LBP variant, as well as various half-life enhancing molecules known in the art such as immunoglobulin fragments. Both BPI and LBP have been cloned and their DNA and amino acid sequences determined (Figures 3A-3B and 4A-4B, respectively). The DNA and amino acid sequences of numerous immunoglobulins are known in the art. For example, the DNA sequence of IgG, IgG_{2a}, and IgG_4 are suitable for use to enhance the half-life of the RENPs of the invention.

Expression of recombinant endotoxin-neutralizing polypeptides

Techniques for obtaining expression of exogenous DNA or RNA sequences in a host cell are known in the art (see, for example, Sambrook et al., *supra*; hereby incorporated by reference with respect to methods and compositions for eukaryotic and prokaryotic expression of a DNA encoding an RENP). Where the transformed cell is a prokaryotic host cell, the preferred host is *Escherichia coli*. Where the transformed cell is a eukaryotic host cell, preferably the host cell is a mammalian cell or a yeast cell. Preferably, the mammalian host cell is a Chinese Hamster Ovary (CHO) cell. Preferably, the yeast host cell is of the genus *Pichia*, more preferably a strain of *Pichia pastoris*.

For prokaryotic expression, the construct should contain at a minimum a bacterial origin of replication

and a bacterial promoter operably linked t the RENP-encoding DNA. For eukaryotic expression, the construct should contain at a minimum a eukaryotic promoter operably linked to a DNA of interest, which is in turn operably linked to a polyadenylation sequence. The polyadenylation signal sequence may be selected from any of a variety of polyadenylation signal sequences known in the art. Preferably, the polyadenylation signal sequence is the SV40 early polyadenylation signal sequence. The eukaryotic construct may also include one or more introns, which can increase levels of expression of the DNA of interest, particularly where the DNA of interest is a cDNA (e.g., contains no introns of the naturally-occurring sequence). Any of a variety of introns known in the art may be used. Preferably, the intron is the human β-globin intron and inserted in the construct at a position 5′ to the DNA of interest.

Purification of RENPs

Purification of the RENPs of the invention can be performed according to any of a variety of protein purification techniques known in the art including gel electrophoresis, immunoprecipitation, ion exchange chromatography, affinity chromatography, or combinations thereof (see, for example, Guide to Protein Purification, Deutscher, ed., Academic Press, Inc., San Diego, CA, 1990). Preferably, purification of RENPs is accomplished by a combination of column chromatographic techniques. For example, RENPs can be purified using a four-step purification procedure using 1) a cation exchange column (e.g., CM Sepharose), 2) an anion exchange column (e.g., Fast Q Sepharose), 3) a second cation exchange column (e.g., CM Sepharose), and 4) a gel filtration sizing column (e.g., Sepharose CL6B).

Pharmaceutical compositions

The RENPs of the invention can be formulated as an active ingredient in a pharmaceutical composition. In general, the pharmaceutical composition contains a therapeutically effective amount of an RENP and a pharmaceutically acceptable carrier. The pharmaceutical composition can contain one or more RENPs. The amount of RENP which constitutes a therapeutically effective amount will vary according to the time of administration (e.g., therapeutic or prophylactic administration), the disease or condition to be treated, the route of administration, and various patient-dependent factors such as age, weight, gender, and severity of disease. Specific therapeutically effective amounts appropriate for administration are readily determined by one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th ed., Gennaro, ed., Mack Publishing Company, Easton, PA, 1990).

Pharmaceutically acceptable carriers suitable for use in the RENP-containing pharmaceutical compositions of the invention are well known to those skilled in the art. Selection of the pharmaceutically acceptable carrier will depend upon a variety of factors including the RENP to be administered, the route of administration, and the condition to be treated.

Pharmaceutically acceptable carriers suitable for use with the RENPs of the invention include, but are not limited to, 0.01-0.1 M and preferably 0.05 M succinate buffer or 0.8% saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Further, pharmaceutically acceptable carriers may include detergents, phospholipids, fatty acids, or other lipid carriers. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive

oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils.

Pharmaceutically acceptable carriers for use with the RENPs of the invention include lipid carriers. A lipid carrier is any lipid-soluble substance which inhibits protein precipitation and in which the proteins of the subject invention are soluble. Lipid carriers can be in the form of sterile solutions or gels, or can be detergents or detergent-containing biological surfactants. Examples of nonionic detergents include polysorbate 80 (also known as TWEEN 80 or polyoxyethylenesorbitan monooleate). Examples of ionic detergents include, but are not limited to, alykltrimethylammonium bromide. Exemplary lipid carriers and methods for solubilizing BPI, and thus which can be used in pharmaceutical compositions containing an RENP of the invention, are described in USPN 5,234,912, incorporated herein by reference.

Where the pharmaceutically acceptable carrier is a lipid carrier, the lipid carrier may be a liposome. A liposome is any phospholipid membrane-bound vesicle capable of containing a desired substance, such as BPI or BPI variant, in its hydrophilic interior. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives, other pharmaceutically active compounds, and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

Disease conditions amenable to treatment with RENPs

Various disease conditions are amenable to treatment using the recombinant LPS-neutralizing proteins of the invention. In general, any condition of a mammalian subject (e.g., human, canine, feline, or bovine, preferably a human) which is associated with a toxic effect of endotoxin can be treated by administration of the RENPs of the invention. Endotoxin-related disorders amenable to treatment include, but are not limited to, endotoxin-related shock, endotoxin-related disseminated intravascular coagulation, endotoxin-related anemia, endotoxin-related thrombocytopenia, endotoxin-related adult respiratory distress syndrome, endotoxin-related renal failure, endotoxin-related liver disease or hepatitis, systemic immune response syndrome (SIRS) resulting from Gram-negative infection, Gram-negative neonatal sepsis, Gram-negative meningitis, Gram-negative pneumonia, neutropenia and/or leucopenia resulting from Gram-negative infection, hemodynamic shock and endotoxin-related pyresis. Endotoxin-related pyresis is associated with certain medical procedures, such as, for example, trans-urethral resection of the prostate, and gingival surgery. The presence of endotoxin may result from infection at any site with a Gram-negative organism, or conditions which may cause ischemia of the gastrointestinal tract, such as hemorrhage, or surgical procedures requiring extracorporal circulation. The important role of endotoxin in hemorrhage (with endogenous LPS translocation from the gut), trauma, and sepsis is well known. One skilled in the art can recognize additional conditions which can be treated using the therapy of the invention.

The recombinant, endotoxin-neutralizing proteins of the invention can also be administered to a patient

prophylactically, e.g. to a patient at risk of an endotoxin-related disorder. For example, the RENPs can be administered to a patient who has a Gram-negative infection and is at risk of bacteremia, but who has not yet exhibited symptoms associated with the toxic effects of endotoxin. The RENPs can also be administered prior to surgery where the risk of introduction of endotoxin into the patient is substantial. One of ordinary skill in the art can readily recognize other instances in which prophylactic administration of a RENP is appropriate. The conditions which identify an individual as being at risk of an endotoxin-related disorder are well known in the art.

Administration of RENPs

The recombinant, LPS-binding protein of the invention may be administered using various methods well known in the art. U.S. Pat. Nos. 5,171,739; 5,308,834; and 5,334,584; each incorporated herein by reference, describe methods and compositions for administration of BPI, and thus can provide additional guidance for administration of the RENPs of the invention. For example, the recombinant, LPS-binding protein can be administered by injection or inhalation. Administration by injection can be an intravenous, intramuscular, or subcutaneous route, or by direct injection directly into a site of infection (e.g., tissue or body cavity). Preferably, injection is intravenous. Administration by inhalation is accomplished by delivery of the RENP to the lungs via an aerosol delivery system or via direct instillation. The aerosol may be nebulized. Various devices and methods for aerosol drug delivery are well known in the art. Methods for determining the appropriate route of administration and dosage are generally determined on a case-by-case basis by the

attending physician. Such determinations are routine to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th ed., Gennaro, ed., Mack Publishing Company, Easton, PA, 1990).

Therapeutically effective amounts of an RENP can be determined according to methods well known to those skilled in the art. Specific dosages will vary according to a variety of factors, including the time of administration (e.g., therapeutic or prophylactic administration), the disease or condition to be treated, the route of administration, the RENP to be administered, and various patient-dependent factors such as age, weight, gender, and severity of disease. The specific dosage appropriate for administration is readily determined by one of ordinary skill in the art according to the factors discussed above (see, for example, Remington's Pharmaceutical Sciences, 18th ed., Gennaro, ed., Mack Publishing Company, Easton, PA, 1990). In addition, the estimates for appropriate dosages in humans may be extrapolated from determinations of the *in vitro* LPS binding affinity of the RENP used, the amount of the RENP effective to inhibit cytokine production by mononuclear cells *in vitro*, the amount of RENP effective to provide protection to LPS challenge, and/or various other *in vitro* and *in vivo* assays indicative of the biological activity of the RENP.

In general, the amount of RENP administered is an amount effective to bind LPS and thereby inhibit the undesirable biological activities associated with LPS including monocyte and neutrophil activation, TNF production, cytokine production, and other biological phenomena triggered by LPS in endotoxin-related disorders. Preferably, the amount of RENP administered is an amount effective to bind LBP and inhibit LPS-mediated stimulation of neutrophils and mononuclear cells.

In therapeutic administration of the RENPs of the invention, an effective amount of an RENP is an amount effective to bind to LPS and thereby inhibit LPS-mediated stimulation of neutrophils and mononuclear cells in a subject having an endotoxin-related disorder. As used herein, "inhibit" means to inhibit at a level which is statistically significant and dose dependent. The terms "statistically significant" and "dose dependent" are well known to those skilled in the art. In general, an effective amount of an RENP in a pharmaceutical composition for treatment of a patient having an endotoxin-related disorder is an amount sufficient to deliver to the subject a recombinant protein of the subject invention at a concentration of between about 0.1 mg/kg of body weight and about 100 mg/kg of body weight, preferably between about 1 mg/kg of body weight and about 10 mg/kg of body weight. Preferably, the RENP(s) is administered by injection, infusion, or as an injected bolus so as to maintain a circulating RENP concentration of about 1-10 μg/ml. The preferred circulating RENP concentration can vary according to a variety of factors, including the LPS binding affinity of the specific RENP(s) administered.

As used herein, a prophylactically effective amount of an RENP in a pharmaceutical composition for the prevention of an endotoxin-related disorder is an amount effective to bind LPS and prevent LPS-mediated biological activity, e.g., LPS-mediated stimulation of monocytes and neutrophils. In general, a prophylactically effective amount of an RENP is an amount of a composition effective to deliver between about 0.1 mg/kg of body weight and about 100 mg/kg of body weight, preferably between about 1 mg/kg of body weight and about 10 mg/kg of body weight, to the patient at risk of an endotoxin-related disorder.

The invention also pr vides an article of manufacture comprising packaging material and a pharmaceutical composition contained within the packaging material. The packaging material includes a label which indicates that the pharmaceutical composition can be used for treating a subject suffering from an endotoxin-related disorder and\or for preventing an endotoxin-related disorder (e.g., inflammation) in a subject. The pharmaceutical composition contains a therapeutically effective and/or prophylactically effective amount of an RENP and a pharmaceutically acceptable carrier.

Assessment of therapy

The efficacy of the therapeutic or prophylactic use of the RENPs of the invention can be determined by monitoring patient symptoms associated with an endotoxin-related disorder. Such symptoms, and methods for monitoring, are well known in the art. For example, where the RENP is used in the treatment of a patient having an endotoxin-related disorder, the effectiveness of the RENP therapy can be assessed by monitoring fever, blood pressure, cytokine levels, and/or LPS levels in the patient's blood stream. The presence of LPS in the blood stream can be assayed as described above. Where the patient is not responding, it may be desirable to increase the dosage of the RENP pharmaceutical composition or, where the patient is not responding favorably, discontinue the RENP regimen.

Detectably-labeled RENPs

Various detectable labels, as well as methods of attachment of such labels to a protein, are well known in the art. Detectable labels can be any molecule recognized in the art as a means for identifying and/or

detecting a protein to which the detectable label is bound. Exemplary "detectable labels" include, but are not limited to radionucleotides, fluorescent moieties, biotin, and antigenic molecules (e.g., a polypeptide which can be specifically bound by an anti-polypeptide antibody). Thus, detectable labels include a portion of a chimeric protein (e.g., a fusion protein or genetically engineered protein) where a portion of the chimeric protein can be detected by, for example, binding of a detectably labeled antibody or other detectably labeled molecule which specifically binds the chimeric protein portion. For example, where the RENP contains a portion of the amino acid sequence of BPI, and an antibody which specifically binds that amino acid sequence of BPI in the context of the RENP is available, the BPI amino acid sequence is the detectable label.

Methods for attaching (e.g., covalently binding) a detectable label to a protein are well known in the art. For example, methods for preparation of ^{125}I-labeled proteins, biotin-labeled proteins, and FITC-labeled proteins are well known. Methods for detectably labeling antibodies are also well known in the art. Methods for the production of antibodies for use in the subject invention (e.g., anti-BPI, anti-LBP, anti-BPI variant, anti-LBP variant, and anti-immunoglobulin fragment antibodies) are well known in the art (see, for example, Antibodies: A Laboratory Manual, Harlow and Lane, eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988).

Detection of LPS *in vitro*

The detectably labeled RENPs of the invention can be used in various methods for the detection of LPS either *in vitro* or *in vivo*. Samples for which *in vitro* LPS detection is desirable include samples from a patient

suspected of having a Gram-negative infection, and samples from a product for use in a medical application (e.g., a recombinant protein solution where the protein was expressed in *E. coli*). Patient samples include samples of any body fluid, preferably blood or urine. Samples may be pre-treated prior to testing by, for example, concentrating the sample, or centrifugation to remove cells and cellular debris.

In general, *in vitro* detection of LPS in a sample suspected of containing LPS (test sample) is performed by contacting the test sample with an RENP of the invention for a time sufficient for the formation of RENP-LPS complexes, and the RENP-LPS complexes detected. The RENP-LPS complexes can be detected by virtue of a detectable label attached to the RENP, or by the binding of an anti-LPS antibody. Binding of the anti-LPS antibody can subsequently be detected by virtue of a detectable label bound to the antibody, or by the binding of a detectably labeled anti-anti-LPS antibody to the RENP-LPS-antibody complex.

The *in vitro* assay can be performed in solution by mixing the sample with a solution containing RENP, separation of RENP-LPS complexes (e.g., by immunoprecipitation), and detection of the RENP-LPS complexes formed, e.g., by virtue of a detectable label bound to the RENP. Alternatively, the *in vitro* assay is performed with RENP bound to a support, e.g., a polymeric substrate such as a microtiter well or a latex bead. Methods for binding proteins to a support are well known in the art. For example, an anti-RENP antibody can be bound to the support and the RENP subsequently bound to the support via binding to the anti-RENP antibody. After binding of the RENP to the support, the sample is then contacted with the support-bound RENP and any LPS in the sample allowed to bind to the RENP. Unbound material is

then washed away, and the RENP-LPS complexes detected by the binding of detectably labeled RENP or detectably labeled anti-LPS antibody.

The *in vitro* assay can also be performed as a competition binding assay. For example, a sample suspected of containing LPS (test sample) and a known amount of detectably labeled RENP are incubated together with a support having LPS bound to its surface. The test sample and the RENP may be preincubated prior to contact with the support-bound RENP. The level of detectably labeled RENP bound to the support in the test sample is compared to the level of detectably labeled RENP bound to the support in a negative control sample (detectably labeled RENP alone). A level of binding of detectably labeled RENP in the test sample which is lower than binding of detectably labeled RENP in the negative control sample is indicative of the presence of LPS in the sample.

In an alternative embodiment, the competition binding assay is performed with support-bound RENP. In this latter assay, detectably labeled LPS (e.g., radiolabeled LPS) is mixed with the test sample suspected of containing LPS, and the samples contacted with the support-bound RENP, and the amount of detectably labeled LPS bound to the support bound RENP detected. A level of detectably labeled LPS bound to the support in the test sample which is significantly lower than the amount of detectably labeled LPS in the negative control sample (radiolabeled LPS alone) is indicative of the presence of LPS in the test sample.

As is apparent from the description above, the *in vitro* LPS assays of the invention can be performed both qualitatively and quantitatively. For example, quantitative assays can be performed by comparing the results btained with the test sample to results obtained

with parallel samples containing known amounts of LPS. Quantitative *in vitro* assays are indicative of, for example, the severity of Gram-negative infection in a patient sample from whom the sample was obtained, or a degree of contamination where the test sample is a fluid for administration to a patient (e.g., where the assay is performed as a step in quality control). One of ordinary skill in the art will appreciate upon reading the above-described *in vitro* assays that numerous variations of these assays can be performed without departing from the spirit or the scope of the invention.

Detection of LPS *in vivo*

Detectably labeled RENPs of the invention, preferably RENPs having an increased LPS binding affinity relative to LBP, can be used as a diagnostic to identify a site of Gram-negative bacterial infection in a patient. For example, a detectably labeled RENP is administered to a patient suspected of having a Gram-negative infection. Preferably, the detectable label is a radionucleotide such as ^{123}I, ^{124}I, ^{125}I, ^{129}I, ^{131}I, or other beta-emitting radionuclide which can be readily detected with either a hand-held gamma radiation detection device or by nuclear medicine scan. Alternatively, the detectable label is a fluorescent molecule or other visually detectable label which can be visualized during, for example, endoscopy. Detection can be facilitated by increasing the ratio of detectable label to RENP.

The detectably labeled RENP is administered to the patient in an amount sufficient for binding of the RENP to the suspected infection site and detection of the detectable label. The detectably labeled RENP can be administered by injection, preferably by either intravenous injection or by direct injection into the body cavity or tissue suspected of containing the

inf ction site. In general, the amount of detectably labeled RENP administered will vary with according to numerous variables including the RENP and detectable label used, the location of the suspected site of infection, the route of administration, and various patient factors including size, weight, age, and suspected severity of the disease.

After administration, the detectably labeled RENP is allowed to circulate to reach the site of infection and/or incubate over the suspected site of infection. Bound detectably labeled RENP is detected using methods appropriate for the label used. For example, where the detectable label is a radionucleotide, bound RENP is detected using a radiation detecting device. Using this method, the site and the extent of a Gram-negative infection can be determined. Where desirable, the detectably labeled RENPs can be used to label a site or sites of infection which can then be imaged using any of a variety of imaging techniques known in the art (e.g., X-ray, CAT scan, MRI, or PET scan).

LPS decontamination using RENPs

The RENPs of the invention can also be used in the decontamination of a product prior to its medical application. For example, where a recombinant protein has been produced by expression in *E. coli*, a solution containing the recombinant protein can be applied to a support having bound RENP (e.g., an affinity column). LPS in the solution binds to the RENP bound to the support, and the LPS-free solution is collected. If necessary, the decontamination step can be repeated multiple times until an acceptably low amount of LPS (e.g. 0 to 0.001 ng/ml is detected in the solution. Such decontamination procedures using the RENPS of the

invention can be used as a final step in quality control of, for example, recombinantly produced pharmaceuticals.

EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to carry out the invention and is not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1: Construction of RENPs

Specific examples of RENPs are described in Table 3, and are additionally designated by a construct name (e.g., NCY103) or lot number of the protein stock.

Table 3

Examples of RENPs

SEQUENCE	CONSTRUCT NAME OR LOT #	DESCRIPTION
BPI	NCY101	Native sequence
$L_{1-197(I43->V)}B_{200-456(N206->D)}$	NCY103	LBP-BPI chimera
$B_{1-199}L_{200-456}$	NCY104	BPI-LBP chimera
$B_{(S351->A)}$	NCY105	Glycosylation site deleted
$B_{(DS200->DP)}$	NCY106	Formic acid cleavage site inserted
$L_{1-199}B_{200-456(S351->A)}$	NCY107	LBP-BPI chimera with glycosylation site deleted
B_{1-199}	NCY108	N-terminal domain of BPI
$B_{(1-190)}$	Lot #159699	N-terminal BPI fragment
$B_{(1-236)}$	Lot #159695	N-terminal BPI fragment
$B_{(1-212)}$	Lot #159693	N-terminal BPI fragment
$B_{1-199}Fc$	NCY110	N-terminal BPI-IgG chimera
$B_{200-456}$	NCY112	C-terminal fragment of BPI
$L_{1-59}B_{60-456}$	NCY114	LBP-BPI chimera
$L_{1-134}B_{135-456}$	NCY115	LBP-BPI chimera
$L_{1-275}B_{278-456}$	NCY116	LBP-BPI chimera
$L_{1-359}B_{360-456}$	NCY117	LBP-BPI chimera
$L_{(1-164)}B_{(200-456)}$	Lot #164325	LBP-BPI chimera
$L_{(1-175)}B_{(200-456)}$	Lot #164326	LBP-BPI chimera
$L_{1-197}B_{200-456}$	NCY118	LBP-BPI chimera
$B_{(F61->C)}$	NCY119	Cysteine insertion
$B_{(C132->A)}$	NCY120	Cysteine substitution

$B_{(C132->S)}$	NCY121	Cysteine substitution
$B_{(C135->S)}$	NCY122	Cysteine substitution
$B_{(C175->S)}$	NCY123	Cysteine substitution

Table 3 (continued)

SEQUENCE	CONSTRUCT NAME OR LOT #	DESCRIPTION
$B_{(C132->A)(C135->S)(C175->S)}$	NCY124	Multiple cysteine substitution
$B_{(1-132->A)(C135->S)(C175->S)}$	NCY125	Multiple cysteine substitution
$L_{(1-134)}B_{(136-361)}L_{(360-456)}$	NCY133	LBP-BPI chimera
$L_{(1-134)}B_{(136-275)}L_{(274-456)}$	NCY134	LBP-BPI chimera
$L_{(1-198)}B_{(202-275)}L_{(274-456)}$	NCY135	LBP-BPI chimera
$L_{(1-198)}B_{(202-361)}L_{(360-456)}$	NCY136	LBP-BPI chimera
$B_{(1-41)}L_{(42-199)}B_{(200-456)}$	Lot #162303	BPI-LBP-BPI chimera
$B_{(1-190)(C178->A)}$	Lot #162305	N-terminal BPI fragment with cationic substitution
$B_{(K27->S)(K30->L)(K33->T)(K42->R)(K44->P)(K48->R)(R59->H)}$ (B_{CAT7})	NCY137	Cationic Substit. (7)
$B_{(K77->S)(K86->R)(K90->R)(R96->S)(K118->L)(K127->R)}$ (B_{CAT6})	NCY138	Cationic Substit. (6)
$B_{(K148->G)(K150->D)(K160->N)(K161->Q)(R167->Q)(K169->V)(K177->M)(K185->D)(K198->E)}$ (B_{CAT9})	NCY139	Cationic Substit. (9)
$B_{(K77->S)(K86->R)(K90>R)(K96->S)(K118->L)(K127->R)(K148->G)(K150->D)(K160->N)(K161->Q)(R167->Q)(K169->V)(K177->M)(K185->D)(K198->E)}$ (B_{CAT15})	NCY140	Cationic Substit. (15)
$L_{(S77->K)(R86->K)(R90->K)(S96->K)(L118->K)(R126->K)}$ (L_{CAT6})	NCY141	Cationic Repl. (6)

$L_{(G147->K)(D148->K)(N158->K)(Q159->K)(Q165->R)(V167->K)(M175->K)(D183->K)(E196->K)}$ (L_{CAT9})	NCY142	Cationic Repl.(9)
$L_{(S77->K)(R86->K)(R90->K)(S96->K)(L118->K)(R126->K)(G147->K)(D148->K)(N158->K)(Q159->K)(Q165->R)(V167->K)(M175->K)(D183->K)(E196->K)}$ (L_{CAT15})	NCY143	Cationic Repl. (15)
$L_{(1-198)}B_{(201-456)}Fc$	NCY144	LBP-BPI-IgG chimera

Table 3 (continued)

LBP	NCY102	native sequence
L_{1-199}	NCY109	N-terminal LBP fragment
$L_{1-199}Fc$	NCY111	LBP-Ig chimera
$L_{200-458}$	NCY113	C-terminal LBP fragment
$L_{(A132\rightarrow C)}$	NCY126	Cysteine insertion
$L_{(C61\rightarrow F)}$	NCY127	Cysteine substitution
$L_{(C61\rightarrow S)}$	NCY128	Cysteine substitution
$L_{(C135\rightarrow S)}$	NCY129	Cysteine substitution
$L_{(175\rightarrow S)}$	NCY130	Cysteine substitution
$L_{(C61\rightarrow F)(C135\rightarrow S)(C175\rightarrow S)}$	NCY131	Multiple cysteine substitution
$L_{(C61\rightarrow S)(C135\rightarrow S)(C175\rightarrow S)}$	NCY132	Multiple cysteine substitution

The proteins encoded by the LBP and $L_{1-359}B_{360-456}$ constructs facilitated the LPS-mediated cellular response, indicating that LBP amino acid residues 275-359 are required for this LBP activity.

The cDNA sequences of BPI and LBP are shown in Figures 3A-3D and 4A-C, respectively, with each nucleotide designated numerically. DNA encoding the RENPs can be prepared using a variety of techniques well known in the art, including protein fusion techniques, site-directed mutagenesis, and PCR (see, for example, Sambrook et al., *supra*; Zoller, M.J., et al., Methods Enzymol. 154:329 (1987)). For example, in the construction of the RENP $L_{1-197}B_{200-456}$, the sequence "$ATAGAT_{723}$" and "$ATTGAC_{700}$" was chosen as a convenient site to insert a ClaI restriction site (ATCGAT) by which to recombine portions of both BPI (former) and LBP (latter). Oligonucleotide primers were designed which overlap this region but contain the ClaI sequence, and

were synthesized on an ABI 380B synthesizer (Applied Biosystems Inc., Foster City, CA). Additional primers were designed to bind to the 5′ and 3′- ends of both molecules, which primers contained NheI (5′) and XhoI (3′) restriction sites for insertion into the vector. These primers were used to amplify portions of the cDNA molecules encoding amino acid residues 1-199 (A) and 200-456 (B) of LBP and BPI by cyclic DNA amplification. The resulting DNA fragments were digested with the appropriate restriction enzymes and then purified by gel electrophoresis.

Example 2: Mammalian Expression

In order to produce BPI, LBP, or RENPs of the invention in mammalian cells, the cDNA sequences were inserted into a suitable plasmid vector. A suitable vector for such an application is pSE, which contains the origin of replication and early and late promoters of SV40, followed by multiple insert cloning sites, followed by the termination sequences from the hepatitis B surface antigen gene. An origin of bacterial DNA replication, and the genes encoding ampicillin resistance and dihydrofolate reductase were also included in the plasmid for production of large amounts of DNA using bacterial host cells. Similar vectors have been used to express other foreign genes (Simonsen et al., *Biologicals* 22:85 (1994). Another suitable vector, particularly for rapidly obtaining small quantities of RENPs was pCIP4 (Invitrogen Corp., San Diego, California). pCEP4 contains a CMV promoter, followed by multiple insert cloning sites, followed by SV40 termination sequences. Also contained within the plasmid are an origin of bacterial DNA replication, and the genes encoding resistance to ampicillin and hygromycin B. With pCEP4 and pSE, the same insert cloning sites as pSE for easy

insert shuttling betwe n the vect rs were used. Once introduced into mammalian cell hosts, this specialized plasmid replicates as an episome, allowing semistable amplification of introduced DNA sequences. The high gene copy number is maintained through the selective pressure of culture in the presence of hygromycin B.

A second expression system (EBV/293) was used to rapidly obtain small quantities of recombinant proteins of the subject invention when useful. This system was constructed to use the same insert cloning sites as pSE for easy insert shuttling, but utilized the Epstein-Barr virus promoter (EBV) to drive heterologous expression (pCEP4). Once introduced into mammalian cell hosts, this specialized plasmid replicates as an episome, allowing semistable amplification of introduced DNA sequences. The high gene copy number is maintained through the selective pressure of culture in the presence of hygromycin plus G418. Similar expression systems are commercially available (e.g., Invitrogen, Inc., San Diego, CA).

Vector DNA was prepared for acceptance of BPI cDNA by digestion with Nhe I and Xho I, and was subsequently dephosphorylated by treatment with alkaline phosphatase. The prepared fragments encoding BPI, LBP, or an RENP were ligated into pSE or pCEP4, and the resulting recombinant colonies were screened by agarose gel electrophoresis. Subsequently, the DNA sequences were confirmed by standard enzymatic sequencing methods (e.g., Sanger, 1974).

Expression plasmid DNA purified by either CsCl gradients with Plasmid or Midi Kits (Qiagen, Chatsworth, California) was used to transform Chinese hamster ovary strain DUXB11 (pSE) and 293-EBNA cells (Invitrogen Corp., San Diego, California) (pCEP4). Transfection was performed using lipofectin (B thesda, Research Labs,

Gaithersberg, MD) by standard methods. The resulting transformed cells were selected in GHT minus medium (DUKXB11s) or in REM and 10% calf serum (293s). For the DUKXB11s, clones were selected and were passed through sequential rounds of culture in increasing concentrations of methotrexate in order to amplify the DHFR gene and associated heterologous genes. Supernatants from transfected cells, either mixed populations or clones derived from the mixed population, were assayed for RENPs by ELISA using antibodies specific for BPI, LBP, or immunoglobulin as appropriate.

Example 3: Yeast Expression

BPI and $L_{1-197}B_{200-456}$ were successfully expressed in the methylotrophic yeast *Pichia pastoris*. Pichia was chosen as a suitable expression system for BPI and RENPs due to its lack of LPS (endotoxin to which BPI and RENPs bind) and its ability to produce high levels of mammalian proteins.

Pichia pastoris strain GS115 (Invitrogen, San Diego, California) was transformed with plasmids encoding BPI and $L_{1-197}B_{200-456}$, and transformed colonies were selected according to the procedures outlined by Invitrogen (A Manual of Methods for Expression of Recombinant Proteins in Pichia pastoris, Version 1.5, Invitrogen, San Diego, California). For both BPI and $L_{1-197}B_{200-456}$, protein was secreted into the medium in a small-scale batch fermentation run. 116 ng/ml were secreted for the one BPI construct assayed, and 14, 11, and 10 ng/ml were secreted for the three constructs $L_{1-197}B_{200-456}$ constructs assayed. Secretion was assayed by enzyme-linked immunosorbant analysis (ELISA). The majority of protein for both constructs was not secreted, as shown by Western blot analysis with a polyclonal anti-BPI antibody (INVN 1-2) (prepared by conventional

t chniques by injecting rabbit with BPI) and alkaline phosphatase-conjugated goat anti-rabbit antibody. The Western blot is shown in Figure 18.

Purified BPI from Chinese Hamster ovary cells (CHOs) was used as a positive control (lane 12). In lane 1 a sample from untransformed GS115 cells served as a negative control. The antibodies did not recognize any proteins from the untransformed GS115 cells. The next three lanes (2-4) were samples from colonies transformed with the construct for BPI and the last 6 lanes (5-10) were samples from colonies transformed with the construct for $L_{1-197}B_{200-456}$. The amount of intracellular BPI or $L_{1-197}B_{200-456}$ expressed in the batch fermentation run, based on the amount of standard BPI loaded, was roughly 100 μg/ml of medium for the BPI and $L_{1-197}B_{200-456}$ colonies.

Example 4: Protein Purification

BPI was purified from conditioned media using the following four-step purification. BPI was captured on CM Sepharose (Pharmacia LKB Biotechnology). The column was washed in 50 mM Tris pH 7.4, and protein was eluted with 50mM Tris buffer pH 7.4 + 1 M NaCl. The eluate was diluted 10X with 50mM Tris pH 8.5, run over Fast Q Sepharose, and the flow-through was collected. BPI was re-captured on CM Sepharose and again eluted as before. Buffer exchange into 10 mM Succinate + 110 mM NaCl pH 6 was performed using Sepharose CL6B (Pharmacia LKB Biotechnology). Finally, Tween 20 was added to the formulated material to a final concentration of 0.05%.

LBP (NCY102) was captured from cell culture medium on Fast S Sepharose (Pharmacia). The column was washed with 50 mM Tris pH 7.4, and protein was eluted using 50mM Tris pH 7.4 + 1 M NaCl. The eluate was diluted 10X in 50mM Tris pH 8.5 and run over HiLoad Q Sepharose

(Pharmacia). Protein was eluted with a 0-1 M NaCl gradient in 50mM Tris pH 8.5. Appropriate fractions were pooled according to migration on SDS PAGE electrophoresis. LBP concentration was diluted to 4.0 mg/ml, and the pH was adjusted to 7.0 with 100 mM HCl.

$L_{1-197(I43->V)}B_{200-456(N206->D)}$ was purified from cell culture medium using the same method described for LBP.

$B_{1-199}L_{200-456}$ and $B_{(S351->A)}$ were purified using the same protocol as for BPI, except that the size exclusion step was omitted.

$L_{1-59}B_{60-456}$, $L_{1-134}B_{135-456}$ and B_{CAT6} were captured on a Poros II HS cation exchange column (PerSeptive Biosystems, Cambridge, MA) at pH 7.4. The column was washed with 20 mM HEPES buffer at pH 7.5, and eluted with 20 mM HEPES pH 7.5 with 1 M NaCl. The eluate was diluted 5X in 20 mM HEPES pH 7.5 and applied to a Poros HQ anion exchange column (PerSeptive) with the flow-through applied directly to a POROS II HS column. The POROS II HS column was eluted with 3.3 mM acetate, 3.3mM MES and 3.3 mM HEPES, pH 6.0 with a 0-1 M NaCl gradient.

$L_{1-359}B_{360-456}$ and $L_{(1-198)}B_{(201-456)}$Fc were captured from conditioned medium at pH 7.4 on a Poros II HS column. The column was washed with 20mM HEPES buffer at pH 7.5, and eluted with 20mM HEPES pH 7.5 + 1 M NaCl. The eluate was diluted 10X with 20mM HEPES pH 7.5, loaded on a second, smaller Poros II HS column, and eluted with 3.3 mM acetate, 3.3 mM MES and 3.3 mM HEPES, pH 6 with a 0-1 M NaCl gradient.

Example 5: BPI Activity Against N. meningitidis and N. gonorrhoeae

BPI suppresses TNF release by human inflammatory cells in response to lipopolysaccharide (LPS) derived from a wide range of Gram-negative bacterial species. In order to test the activity of BPI against Gram-negative

lipooligosaccharide (LOS) from the pathogenic bacteria Neisseria meningitidis and N. gonorrhoeae, non-viable bacteria were pre-treated with recombinant BPI and incubated with human whole blood for 4 hours at 37°C. Without BPI, N. meningitidis at 105 bacteria/ml stimulated the release of 2.93 ± 0.53 ng/ml of TNF, while N. gonorrhoeae was a more potent stimulator of TNF release: 10_4 bacteria/ml induced 8.23 ± 0.32 ng/ml of TNF. In both cases, 10 μg/ml BPI completely inhibited TNF release. This indicates that BPI is able to bind and detoxify LOS of these organisms, as well as bind LPS. Thus, BPI can be useful as a therapeutic agent against LOS-mediated tissue damage associated with these pathogenic Neisseria species.

Example 6: biotinylated BPI Binding Competition Assays

Competition assays for binding of LPS immobilized on microtiter plates was performed using a modified procedure described by Tobias et al., *J. Biol. Chem.* 264:10867 (1989). Briefly, Immulon 3 microtiter plates (96-well, Dynatech Biotechnology Products, Chantilly, VA) were coated with 1 or 4 μg of S. minnesota R595 Re LPS (LIST Biological Labs, Inc., #304) in 50mM borate pH 9.5-9.8 + 20-25 mM EDTA overnight at 37°C. Blank, non-LPS coated wells were included on each plate and binding to these wells was used to determine non-specific binding. Absorbance values from wells which were not pre-coated with LPS consistently gave optical density readings of less than 0.05. Plates were then washed extensively under running distilled deionized water, then dried at 37°C. Assay wells were blocked for 60 minutes at 37°C with 1-2% very low endotoxin BSA (Sigma, St. Louis, MO) prepared in pyrogen-free Tris-buffered saline (50mM Tris pH 7.4 + 150 mM NaCl). The wells were emptied, and biotinylated BPI was incubated in the

presence or absence of unlabeled BPI or recombinant protein of the subject invention diluted in assay buffer (pyrogen-free TBS + 1 mg/ml low endotoxin BSA, and 0.05% Tween-20) was incubated in the LPS coated and uncoated wells for 2-3 hours at 37°C in a total volume of 100 µl/well. After four washes in assay buffer, plates were developed with streptavidin conjugated to alkaline phosphatase (BioRad, Burlingame, California) followed by 100 µl of PNP substrate solution (Sigma) freshly prepared from two 5 mg tablets dissolved in 10 ml substrate buffer. Substrate buffer is prepared with 24.5 mg $MgCl_2$, 48 ml diethanolamine, brought up to 400 ml, pH adjusted to 9.8 and volume brought up to 500 ml. Absorbances were read at 405 nm on a Vmax kinetic microplate reader (Molecular Devices, Inc., Menlo Park, CA).

The relative LPS binding affinities of BPI, LBP and RENPs were tested in the competitive binding assay described above using 10 ng/ml ^{biotinylated}BPI. In these experiments, BPI inhibited ^{biotinylated}BPI binding to LPS in a concentration-dependent manner (Figure 7). Modest inhibition of ^{biotinylated}BPI-binding was observed using NCY102 (LBP) and $L_{1-197(I43->V)}B_{200-456(N206->D)}$, suggesting that BPI has either a higher affinity for LPS bound to a surface or that LBP and $L_{1-197(I43->V)}B_{200-456(N206->D)}$ bind to a different site on LPS. $B_{1-199}L_{200-456}$, which contains the N-terminal domain of BPI, competed with ^{biotinylated}BPI at similar concentrations as unlabeled BPI, suggesting a similar affinity and binding site.

Competition between either $L_{1-197}B_{200-456}$ (NCY118) or $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103) with biotinylated BPI occurred at similar concentrations, giving overlapping curves (Figure 13A) indicating that the two amino acid differences between these two molecules [$L_{1-197}B_{200-456}$->$L_{1-197(I43->V)}B_{200-456(N206->D)}$: (I43->V) and (N206->D)] had no effect on affinity for immobilized LPS.

$L_{(1-198)}B_{(201-456)}Fc$ (an IgG chim ra consisting of $L_{1-197}B_{200-456}$ linked to human IgG1 Fc constant region of the immunoglobulin molecule) does not have an altered ability to compete with biotinylated BPI (Figure 13A). $L_{1-59}B_{60-456}$ and $L_{1-134}B_{135-456}$ showed a similar affinity for LPS which affinity was very similar to that observed for BPI, suggesting that the region between amino acid residues 1-59 (or 1-134) probably plays a minimal role in LPS binding (Figure 13B). Together with data showing the $B_{1-199}L_{200-456}$ competes effectively with BPI (Figure 7), these results indicate that amino acid residues 134-199 are important structural components of the high-affinity LPS-binding domain of BPI.

The importance of the region between amino acid residues 134 to 197 in LPS affinity was further demonstrated by the markedly reduced affinity of B_{CAT9}, a mutant in which all of the cationic amino acids of the BPI molecule (particularly the cationic residues of BPI amino acids 134-200) are replaced with the corresponding amino acid residues found in LBP. These changes resulted in a molecule with binding affinity for LPS which was more similar to that of LBP than BPI (Figure 13C, and Figure 7). Amino acid residues 360 to 456 of BPI are apparently not involved in LPS binding as demonstrated by the relative inability of $L_{1-359}B_{360-456}$ to displace biotinylated BPI from LPS (Figure 13C). The apparent binding affinity of $L_{1-359}B_{360-456}$ for LPS is similar to that of LBP and B_{CAT9}, which affinity is approximately two orders of magnitude lower than the apparent affinity of BPI for LPS.

Thus, the domain of BPI which participates in binding to immobilized LPS is localized in the N-terminal half of the BPI molecule, since $B_{1-199}L_{200-456}$ has the greatest ability to displace native BPI from LPS coated onto microtiter plates. This domain of BPI has been more

specifically localized to a region between amino acid residues 134-199.

Example 7: Chromogenic LAL Assay

To test the relative abilities of BPI, LBP and RENPs to neutralize LPS *in vitro*, these proteins were tested for inhibitory activity in the chromogenic LAL assay. Briefly, BPI and RENPs (25 µl of 0-200 µg/ml) were pre-incubated for 1 hour at 37°C with 1 EU/ml of E. coli 0111:B4 LPS, (Whitaker Biologicals, Walkersville, MD). The mixtures were then tested for LAL activity using the chromogenic LAL assay kit (Whitaker Biologicals, Walkersville, MD). The results are shown in Figure 8 and Table 4. LPS was neutralized by the various proteins tested in the order of: $B_{(A351->A)} \geq$ BPI > $L_{1-197(I43->V)}B_{200-456(N206->D)}$ > $B_{1-199}L_{200-456}$ > LBP. Several studies were carried out with different lots of each protein and the IC_{50} values were determined. The averaged IC_{50} values are shown in Table 4.

Table 4

LPS Inhibition in the Chromogenic LAL Assay

Product	IC_{50} (μg/ml)	No. of test
—		
$B_{(S351->A)}$	1.5	(n=1)
BPI	5.2 ± 3.3	(n=10)
$L_{1-197(I43->V)}B_{200-456(N206->D)}$	28.0 ± 20.0	(n=4)
$B_{1-199}L_{200-456}$	40.0	(n=1)
LBP	65.0 ± 31.0	(n=4)

These results demonstrate that BPI neutralizes LPS activity in the LAL assay at lower concentrations than LBP. $B_{1-199}L_{200-456}$, which contains the N-terminal domain of BPI, effectively competes with BPI for binding to LPS (see Figure 7) but is a relatively poor inhibitor of LPS in the LAL assay. These results indicate that the N-terminal (LPS-binding) domain of BPI alone does not account for the neutralizing activity of BPI in the LAL assay. $L_{1-197(I43->V)}B_{200-456(N206->D)}$ was a more potent inhibitor than LBP or $B_{1-199}L_{200-456}$, suggesting that the C-terminal domain of BPI plays a very important role in endotoxin neutralization in the LAL assay.

Additional results of LPS neutralizing activity in the chromogenic LAL assay are shown in Table 5. $L_{1-197(I43->V)}B_{200-456(N206->D)}$, $L_{1-59}B_{60-456}$, and $L_{1-134}B_{135-456}$ share the C-terminal half of the BPI molecule, again indicating that this domain plays an important role in LPS-neutralizing activity. Also, these data indicate that the 199-456 region is most important in LPS

neutralization since adding BPI amino acid residues between 136-456 or 60-456 did not improve LPS neutralizing activity. Together with the LPS binding data, these results further indicate that the C-terminal half of BPI is important for neutralization, while the N-terminal sequence is more critical for LPS binding.

Table 5

LPS Inhibition in the Chromogenic LAL Assay

Protein		IC50	n
BPI	Cumulative	1.58 ± 1.58	94
	Lot# 149718	1.57 ± 1.01	54
	Lot# 149719	1.69 ± 0.35	7
	Lot# 149722	1.70 ± 0.28	2
	Lot# 149724	1.41 ± 0.45	45
	Lot# 155794	1.95 ± 0.92	2
LBP	Cumulative	55.92 ± 30.53	8
	Lot# 151281	34.33 ± 7.45	6
	Lot# 151204	77.50 ± 24.45	2
$L_{1-197(I43->V)}B_{200-456(N206->D)}$		Cumulative	
22.86 ± 16.28	54		
	Lot# 151235	25.50 ± 0.71	2
	Lot# 151242	36.50 ± 2.12	2
	Lot# 151274	3.46 ± 2.18	38
	Lot# 159616	8.83 ± 4.91	4
$B_{1-199}L_{200-456}$	Cumulative	24.19 ± 6.42	9
	Lot# 151246	12.50 ± 0.26	3
	Lot# 152658	10.70	1
	Lot# 155737	40.18 ± 34.48	4
B_{1-199}	Cumulative	5.52 ± 5.05	17
	Lot# 151285	1.12 ± 0.00	2
	Lot# 155709	9.73 ± 1.18	3
	Lot# 155779	2.13 ± 0.81	2
$L_{1-59}B_{60-456}$	Lot# 155754	3.64 ± 1.64	5
$L_{1-134}B_{135-456}$	Lot# 155756	5.02 ± 3.11	5
$L_{1-275}B_{278-456}$	Lot# 155791	14.00 ± 2.65	3
$L_{1-359}B_{360-456}$	Lot# 155733	>100	4

Protein		IC50	n
$L_{1-197}B_{200-456}$	Cumulative	12.75 ± 3.54	12
	Lot# 155758	10.25 ± 30.9	8
	Lot# 159619	15.25 ± 5.91	4
B_{CAT6}	Lot# 155785	1.97 ± 0.06	3
B_{CAT9}	Lot# 155762	29.60 ± 23.23	5
B_{CAT15}	Lot# 155788	7.87 ± 2.80	3
$L_{(1-198)}B_{(202-275)}L_{(274-456)}$		Lot# 159649	
>100		3	
$L_{(1-198)}B_{(201-456)}Fc$	Lot# 155760	12.15 ± 6.00	4
L_{1-199}		9.2	1
B_{1-199}		10.1 ± 0.92	5
$L_{(1-134)}B_{(136-275)}L_{(274-456)}$		Lot# 159643	
22.00 ± 15.25		4	

B_{CAT9}, which contains the entire BPI sequence except for nine cationic residues between positions 148 and 197 showed very poor LPS-neutralizing activity, suggesting that these residues are important in LPS-neutralizing activity. Similarly, this compound was relatively ineffective at competing with native BPI for binding to LPS. These cationic residues may permit correct structural conformation of the molecule, since both $L_{1-197(I43->V)}B_{200-456(N206->D)}$ and B_{CAT9} contain the C-terminal domain of BPI, yet $L_{1-197(I43->V)}B_{200-456(N206->D)}$ has potent neutralizing activity while B_{CAT9} does not.

Example 8: Inhibition of FITC-labeled LPS binding to human monocytes

The relative LPs-binding affinities of RENPs of the invention were investigated by examining the abilities of the RENPs to inhibit LPS binding to human

peripheral blood monocytes. Blood collected in acid citrate dextrose-containing VACUTAINER™ tubes (Becton Dickinson, Rutherford, NJ) was diluted 1:4 in Hank's balanced salt solution (HBSS) minus calcium and magnesium (Gibco BRL, Grand Island, MD). Mononuclear cells were isolated using Ficol-Paque (Pharmacia Inc., Piscataway, NJ). Cells were washed three times in HBSS, then brought up to an appropriate volume of RPMI 1640 with glutamine and antibiotics to give approximately 1 X 10^6 cells/ml. To one ml aliquots of cells, FITC-LPS was added to a final concentration of 500 ng/ml. Tubes were closed and incubated at 37°C on a rocking platform. At the end of the incubation, cells were washed twice with PBS with 0.05% Human Serum Albumin and 0.02% sodium azide. FACS analysis of the cells was performed on a FACStar flow cytometer, Immunocytometry System, Becton Dickinson (Mountain View, CA). The monocyte portion of the cell population was determined by side scatter versus forward scatter gating and confirmed by staining a separate aliquot of cells with phycoerythrin-conjugated anti-DR antibody (Becton Dickinson Immunocytometry Systems, Milpitas, CA). Results are reported as logarithmic scale mean fluorescence intensity.

To determine the relative abilities of BPI or $L_{1-197(I43->V)}B_{200-456(N206->D)}$ to inhibit LPS binding to human peripheral blood monocytes, isolated human peripheral blood mononuclear cells were incubated with 10% human serum containing 500 ng/ml FITC-conjugated E. coli 055:B5 LPS in the presence or absence of BPI or $L_{1-197(I43->V)}B_{200-456(N206->D)}$. Binding of FITC-LPS to monocytes could be inhibited by increasing concentrations of both BPI and $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (Figure 9). Thus $L_{1-197(I43->V)}B_{200-456(N206->D)}$ has BPI-like activity, despite the fact that $L_{1-197(I43->V)}B_{200-456(N206->D)}$ contains the N-terminal domain of LBP. These data, along with the

results of the LPS neutralization studies shown in Figure 8, suggest that the C-terminal domains of BPI and LBP, and not the N-terminal domains, determine whether the proteins inhibit or mediate LPS activation of cells.

Further studies were undertaken to determine the effects of BPI, LBP, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ and $B_{1-199}L_{200-456}$ on FITC-labeled LPS binding to peripheral blood monocytes in the presence and absence of serum. In a serum-free FITC-labeled LPS binding system where no LBP is available, FITC-labeled LPS does not bind to cells. In contrast recombinant LBP facilitated LPS binding to cells at concentrations as low as 100 ng/ml. $B_{1-199}L_{200-456}$ also facilitated binding, although to a lesser extent. Neither BPI or $L_{1-197(I43->V)}B_{200-456(N206->D)}$ promoted significant binding of LPS to cells. These data indicate that the C-terminal domain of LBP is active in LPS binding to cells. The N-terminal domain of BPI may exert an inhibitory influence on LPS binding to cells mediated by the C-terminal domain of LBP because $B_{1-199}L_{200-456}$ was less active than LBP.

Normal human serum contains about 1-10 μg/ml LBP. In the presence of 10% autologous serum, BPI and $L_{1-197(I43->V)}B_{200-456(N206->D)}$ potently inhibited FITC LPS binding to monocytes, with BPI showing slightly greater potency. $B_{1-199}L_{200-456}$ had marginal activity, and LBP had no effect (Figure 14A). These data indicate the importance of the BPI C-terminus in this test of LPS neutralization. $B_{1-199}L_{200-456}$, which lacks the C-terminal domain of BPI, is approximately two orders of magnitude less potent at blocking LPS binding. LBP, as expected, had no effect. Thus, BPI can intervene in the sepsis cascade by preventing LPS from binding to monocytes and causing release of TNFalpha.

Example 9: THP-1 Cell TNF Production Assay

THP-1 cells were obtained from the American Tissue Culture Collection (Rockville, MD) and were maintained in REM medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin and 100 μg/ml streptomycin. Cells were passed at 2 x 10^5 cells/ml every 3 days. Responsiveness of THP-1 cells to LPS was induced by culturing the cells for 48 hours in REM medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 units penicillin, 100 μg/ml of streptomycin and 100 nM PMA at 37°C in a humidified atmosphere with 5% CO_2. Cells were cultured in 96-well flat-bottomed tissue culture plates at 1-2 x 10^5 cells per well in a final volume of 200 μl. After 48 hours, adherent cells were washed three times with 200 μl of medium without serum. To 180 μl of medium without serum but with 0.5% HSA, LPS (10 μl at 200 ng/ml) and/or BPI, LBP or other RENPs were added (10 μl at 0-2 mg/ml) and the cells were cultured for an additional 4 hours. After 4 hours, supernatants were transferred to a U-bottomed 96 well plate and the plate was centrifuged (500 x g, 12 min) to pellet any cell debris. Supernatants were then stored in a second plate at -20°C until assayed for TNF by ELISA.

To further identify the regions of BPI which contribute to LPS-neutralizing activity, and the domains of LBP which are responsible for transducing the LPS signal to cells, the abilities of RENPs to replace LBP were compared under serum-free conditions. In these experiments, cells of the promonocytic cell line THP-1 were induced to respond to LPS by culturing for 48 hours with phorbol ester. After induction, cells were stimulated with 19 ng/ml of LPS in the presence or absence of the recombinant protein. In this system, TNF release requires a source of LBP. Data from these experiments (Figure 15) show that only LBP and

$L_{1-359}B_{360-456}$ stimulated TNF release. Thus the domain of LBP responsible for facilitating LPS-induced TNF release is within amino acid residues 199-359. Interestingly, $B_{1-199}L_{200-456}$ did not mediate TNF release in a serum-free system. This may indicate that the N-terminal domain of BPI binds too tightly to LPS to allow transfer of LPS to CD14 on the macrophage surface. Figure 16 shows an additional comparison of TNF production. Because $L_{(1-198)}B_{(202-275)}L_{(274-456)}$ includes LBP domain 274-456 and has activity, the active domain may comprise only residues 274-359.

Example 10: LPS-Induced TNF Release In Whole Blood

Peripheral blood from normal human volunteers was collected in heparin-containing VACUTAINER™ tubes (Becton Dickinson). To one milliliter of whole blood, BPI, a protein of the subject invention, or buffer control was added, followed by 1 ng/ml E. coli 055:B5 refined standard endotoxin (RSE) (Whitaker Bioproducts). Samples were incubated in closed microtubes at 37°C for 4 hours on a rocking platform. At the end of the incubation, samples were centrifuged for 5 minutes at 500 x g at 4°C, the plasma collected and frozen on dry ice until assayed for the presence of cytokines. TNF levels were determined by ELISA using human recombinant TNF (Genentech Inc., South San Francisco, CA) as a standard.

In later studies it was determined that BPI activity in whole blood is inhibited by heparin, and the anticoagulant was changed to citrate. In these experiments, to 120 µl of citrated whole blood, 20 µl of BPI or an RENP (at 0-1 mg/ml) or buffer control, 20 µl of 100 ng/ml of *E. coli* 055:B5 LPS was added to stimulate cells in whole blood samples. These experiments were performed in polypropylene microtiter plates (Costar, Cambridge, MA). After the 37°C incubation step, the

plates were centrifuged 15 min at 500 x g at 4°C and the plasma removed for assaying.

To test the effects of BPI, LBP, and RENPs on LPS activation of TNF production in whole blood, BPI, LBP, $L_{1-197(I43->V)}B_{200-456(N206->D)}$, or $B_{1-199}L_{200-456}$ was mixed with heparinized blood, and LPS was added to the resulting mixture. The blood was incubated for four hours at 37°C, and TNF in the plasma was measured as described, supra. Results are shown in Figure 10. $L_{1-197(I43->V)}B_{200-456(N206->D)}$ was the most potent at blocking TNF release, followed by BPI as the next most potent blocker. $B_{1-199}L_{200-456}$ and LBP had essentially no effect. Thus, in whole blood, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ proved to be the most potent inhibitor of LPS-mediated cytokine stimulation.

When experiments were performed in citrated rather than heparinized whole blood, endotoxin-neutralizing activity of BPI and $L_{1-197(I43->V)}B_{200-456(N206->D)}$ were equivalent (Table 6). In experiments in which recombinant proteins were preincubated with endotoxin before addition to whole blood, the activities of these compounds fell roughly into two groups. BPI, $L_{1-197(I43->V)}B_{200-456(N206->D)}$, B_{1-199}, B_{CAT6}, B_{CAT15}, $L_{1-59}B_{60-456}$, $L_{1-134}B_{135-456}$, and $L_{1-197}B_{200-456}$ possess LPS-neutralizing activity, while LBP, $B_{1-199}L_{200-456}$, L_{1-199}, $L_{1-359}B_{360-456}$ and B_{CAT9} were relatively inactive. Results with $L_{1-275}B_{278-456}$, B_{CAT9}, and $L_{(1-198)}B_{(201-456)}Fc$ were equivocal. When compounds were added to the blood samples immediately prior to LPS, the IC50 values were higher, but the same group of proteins showed activity. These data further indicate the role of the C-terminal region of BPI, demarcated by amino acid residues 200-359, in LPS neutralization in a physiological environment such as whole blood. Because L_{1-199} is not a potent endotoxin-neutralizing protein (see Tables 9 and 11), it

can be concluded that the C-terminal domain of BPI must significantly contribute to the endotoxin-neutralizing activity of $L_{1-197(I43->V)}B_{200-456(N206->D)}$ and $L_{1-197}B_{200-456}$. All compounds which contain this sequence (201-359) are active except B_{CAT9}, which was also inactive in other assays possibly because the cationic amino acid residues which were replaced may be important in configuring the molecule. These data indicate that $L_{1-197(I43->V)}B_{200-456(N206->D)}$ is equivalent to $L_{1-197}B_{200-456}$ in activity, thus implying that the amino acid differences between these two proteins have no affect upon LPS binding affinity.

Table 6

LPS Inhibition in Human Whole Blood

Protein Pre-incubated	IC50 (μg/ml)	n	Not Pre-incubated	IC50 (μg/ml)	n
$L_{1-134}B_{135-456}$	0.15 ± 0.12	3	BPI	2.60 ± 1.52	5
$L_{1-197}B_{200-456}$	2.90 ± 3.59	12	$L_{1-134}B_{135-456}$	3.7 ± 1.60	2
$L_{1-59}B_{60-456}$	0.28 ± 0.25	3	$L_{1-199\backslash 7(143->V)}B_{200-456}$ (N206->D)	7.13 ± 5.92	4
$L_{1-197(143->V)}B_{200-456(}$ N206->D)	0.16 ± 0.11	17	$L_{1-59}B_{60-456}$	15 ± 18.58	2
BPI	0.43 ± 0.49	13	$L_{1-197}B_{200-456}$	26.5 ± 0.71	2
$L_{(1-198)}B_{(201-456)}Fc$	18.00 ± 27.73	3	$L_{1-359}B_{360-456}$	>100	1
$B_{1-199}L_{200-456}$	>100	3	B_{CAT9}	>100	2
$L_{1-359}B_{360-456}$	>100	3	$L_{(1-198)}B_{(201-456)}Fc$	>100	2
B_{CAT9}	11.50 ± 3.54	2*	$B_{1-199}L_{200-456}$	ND	
B_{1-199}	0.73 ± 0.48	6	B_{1-199}	4.0	1
L_{1-199}	>100	2	L_{1-199}	>100	1
B_{CAT15}	0.21 ± 0.26	3			
B_{CAT8}	0.27 ± 0.25	2			
$L_{(1-134)}B_{(136-275)}L_{(274-456)}$	2.0	1			
$L_{(1-198)}B_{(202-275)}L_{(274-456)}$	5.27 ± 5.83	3			
$L_{1-275}B_{278-456}$	38.10 ± 53.64	3			

*Two additional values for B_{CAT9} were >100.

Example 11: Mouse Serum Half-Life Assay

CD-1 mice weighing approximately 20 grams were injected with 5 mg/kg body weight BPI, LBP, or RENPs (1 mg/ml) at time zero. In heparinized (or later EDTA-containing) tubes, blood was collected from the retroorbital plexus from three animals for each time point tested. A typical blood collection schedule was 5, 10, 15, 30, 45, 60, 90, 120, 240, and 360 minutes. The EDTA anticoagulated blood was centrifuged for about 10 min at 1000 x g and the supernatant plasma removed and stored frozen on dry ice until tested. Levels of BPI, LBP, or RENP in the plasma samples were determined by ELISA using the appropriate protein as the standard.

A potent anti-endotoxin therapeutic should not only neutralize endotoxin, but should also have the capacity to clear endotoxin from the circulation. The circulating levels of radioactively labeled ^{125}I-BPI were measured in the mouse in the presence and absence of endotoxin (Table 7). In the absence of endotoxin, the elimination (alpha) phase for ^{125}I-BPI was less than two minutes. In the presence of LPS, the alpha phase was extended to 6.2 minutes. ^{125}I-LPS alone has a single phase distribution (beta) with a half-life of about 101 minutes. When ^{125}I-LPS and unlabeled BPI were administered, a 6.2 minute elimination (alpha) phase was observed, indicating that elimination was remarkably facilitated by BPI.

Table 7

Serum Half-Life of BPI and LPS in the Mouse

Test Article	$t_{1/2}$alpha	$t_{1/2}$beta
^{125}I-BPI	1.6	103.0
^{125}I-BPI + LPS	6.3	72.0
^{125}I-LPS	---	101.0
^{125}I-LPS + BPI	6.2	114.0

In order to determine whether the very short circulating half-life of BPI could be extended by molecular engineering, the circulating half-lives of BPI, LBP, $B_{1-199}L_{200-456}$ and $L_{1-197(I43->V)}B_{200-456(N206->D)}$ were compared (Figure 11). Using both labeled and unlabeled material, it was observed that the circulating half-life of BPI in the mouse is remarkably short. This may be a result of the highly cationic nature of BPI having a predicted pI of 10.6. LBP, normally present in the circulation at concentrations of 10 μg/ml, has a predicted pI of about 6.8. As expected, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (LBP-BPI chimera lacking BPI cationic residues) has a markedly longer circulating half-life than $B_{1-199}L_{200-456}$ (BPI-LBP chimera having BPI cationic residues). Figure 11 shows that $L_{1-197(I43->V)}B_{200-456(N206->D)}$ indeed has a longer half-life than BPI. $B_{1-199}L_{200-456}$, with the N-terminal domain of BPI, had an even shorter half-life than that of BPI. Thus, the N-terminal domain of BPI appears to play a major role in its short circulating half-life.

Further pharmacokinetic studies were performed in which recombinant proteins of the subject invention were administered to CD-1 mice at a 5 mg/kg bolus dose. Results of these experiments are shown in Figures

17A-17H. At 5 mg/kg, the circulating half life of $B_{1-199}L_{200-456}$ was similar to that of BPI. $L_{1-197(I43->V)}B_{200-456(N206->D)}$ and $L_{1-197}B_{200-456}$ had overlapping elimination curves and again indicating that these two molecules are equivalent with respect to their biological activities. $L_{1-197(I43->V)}B_{200-456(N206->D)}$ and B_{1-199} persisted in the circulation significantly longer than BPI or $B_{1-199}L_{200-456}$, but not as long as the serum protein LBP. Comparison of the elimination curves of $L_{1-59}B_{60-456}$, $L_{1-134}B_{135-456}$ and B_{CAT9} revealed that the N-terminus of LBP plays a role in extending circulating half-life. $L_{1-59}B_{60-456}$ circulates slightly longer than BPI, and contains the least LBP sequence of any of the recombinant proteins tested (amino acid residues 1-59). $L_{1-134}B_{135-456}$ was cleared somewhat more slowly, indicating that LBP amino acid residues 60-134 of LBP impart a longer circulating half-life. In contrast, the cationic residues of BPI between 134-199 shorten the half-life, since in B_{CAT9}, where the cationic residues in this region were replaced with the corresponding residues of LBP, the half-life was similar to that of $L_{1-134}B_{135-456}$. Including more LBP residues in the N-terminal domain further extends the half life. If amino acid residues 199-359 of LBP are added ($L_{1-359}B_{360-456}$), the half-life is longer, but not quite as long as that of LBP. Likewise $L_{(1-198)}B_{(202-275)}L_{(274-456)}$ (with LBP domain 1-198 and 274-456) has a relatively long t1/2. These results indicate that the more "LBP-like" the molecule is, the longer it circulates. In addition, combining an Ig fragment Fc with $L_{1-197(I43->V)}B_{200-456(N206->D)}$ gives the longest half life.

Example 13: Mouse Endotoxin Challenge Assay

Female CD-1 mice were injected in the lateral tail vein with a LD_{100} dose (25-35 mg/kg) of Salmonella abortus

equi endotoxin, which was followed by an injection of BPI, RENP, or vehicle control into the opposite lateral tail vein at the indicated time. Protein injection concentrations varied and provided doses of 0.5, 1, and 5 mg/kg. Use of vehicle control illustrated the lethality of the endotoxin challenge in the test animal. Animals were observed for mortality at 24, 28, and 72 hours. Preliminary studies showed that mortality does not change from day three to day seven or beyond.

The efficacies of BPI, LBP, $L_{1-197(I43->V)}B_{200-456(N206->D)}$, $B_{1-199}L_{200-456}$ and $B_{(S351->A)}$ against lethal endotoxin challenge in mice were compared (Tables 8-10). The efficacies of $L_{1-197(I43->V)}B_{200-456(N206->D)}$, $L_{1-197}B_{200-456}$, $L_{1-59}B_{60-456}$, $L_{1-134}B_{135-456}$, $L_{(1-198)}B_{(201-456)}Fc$, $L_{1-275}B_{278-456}$, $L_{1-359}B_{360-456}$, B_{CAT9}, B_{CAT6}, and B_{CAT15} against lethal endotoxin challenge in mice were also compared (Table 11). When each protein was given within two minutes after lethal endotoxin challenge, BPI, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ and $B_{(S351->A)}$ had similar potency, whereas LBP and $B_{1-199}L_{200-456}$ showed minimal protection. The marginal protective effects of LBP and $B_{1-199}L_{200-456}$ since these agents do not block the inflammatory signal of LPS acting on human cells *in vitro* (Figure 10).

Table 8

Mouse Endotoxin Challenge
Comparison of BPI, LBP (NCY102), and $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103)

Drug	Dose	% Survival (n=10)
Control	0 mg/kg	0%
BPI	5 mg/kg	60%
	1 mg/kg	40%
LBP	5 mg/kg	30%
	1 mg/kg	20%
$L_{1-197(I43->V)}B_{200-456(N206->D)}$	5 mg/kg	60%
	1 mg/kg	50%

Table 9

Mouse Endotoxin Challenge
Comparison of BPI, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ and $B_{(S351->A)}$

Drug	Dose	% Survival (n=10)
Control	0 mg/kg	0%
BPI	5 mg/kg	80%
$L_{1-197(I43->V)}B_{200-456(N206->D)}$	5 mg/kg	100%
$B_{(S351->A)}$	5 mg/kg	90%

Table 10

Mouse Endotoxin Challenge
Comparison of BPI and $B_{1-199}L_{200-456}$ (NCY104)

Drug	Dose	% Survival (n=10)
Control	0 mg/kg	40%
BPI	10 mg/kg	100%
	2 mg/kg	100%
	0.4 mg/kg	70%
$B_{1-199}L_{200-456}$	10 mg/kg	60%
	2 mg/kg	60%
	0.2 mg/kg	50%

Table 11

Survival in CD-1 Mice Following
Lethal Endotoxin Challenge

Panel A

	Survivors/n	% Survival	p (vs.control)
BPI	40/50	80.00	< 0.001
$L_{1-197(I43->V)}B_{200-456(N206->D)}$	17/20	85.00<	0.001
$L_{1-197}B_{200-456}$	16/20	80.00	< 0.001
$L_{1-59}B_{60-456}$	13/20	65.00	< 0.001
$L_{1-134}B_{135-456}$	13/20	65.00	< 0.001
$L_{(1-198)}B_{(201-456)}Fc$	5/10	50.00	0.002
$L_{1-359}B_{360-456}$	2/10	20.00	0.149
B_{CAT6}	9/10	90.00	< 0.001
B_{CAT9}	1/10	10.00	0.442
$L_{1-275}B_{278-456}$	0/10	0	--
B_{CAT15}	6/10	60.0	< 0.05
Control	1/30	3.30	--

Panel B

	Dose mg/kg	Survivors (n=20)	% Survival	p (vs. control)*
BPI				
	5	13	65	< 0.001
	1	9	45	0.001
	0.5	6	30	0.02
$L_{1-197(I43->V)}B_{200-456(N206->D)}$				
	5	18	90	<0.001
	1	12	60	<0.001
	0.5	9	45	0.001
B_{1-199}				
	5	3	15	NS
	1	0	0	NS
	0.5	1	5	NS

* Fisher's Exact Test

$L_{1-197(I43->V)}B_{200-456(N206->D)}$ was markedly more effective than BPI when given more than an hour before or after LPS (Figure 12). These results indicate that the longer circulating half-life of $L_{1-197(I43->V)}B_{200-456(N206->D)}$, or perhaps the increased ability of $L_{1-197(I43->V)}B_{200-456(N206->D)}$ to inhibit endotoxin in whole blood, has a dramatic effect on $L_{1-197(I43->V)}B_{200-456(N206->D)}$ efficacy *in vivo*.

Further experiments were performed to assess the LPS-neutralizing activities of recombinant proteins of the subject invention *in vivo*. In these experiments, a lethal LPS challenge was administered at time zero, followed immediately by a 5 mg/kg bolus injection of recombinant protein.

Example 12: BPI Reduction of LPS-Induced Cytokine Function and Mortality in Rats

The potential effect of BPI against LPS related cytokine formation and mortality was investigated in rats with either (a) hemorrhagic shock (bled to lower pressure to 30-35 mmHg mean arterial pressure for 90 minutes, followed by reinfusion of shed blood and an equal volume of Ringer's over 30 minutes), or (b) endotoxic shock (caused by 100 μg LPS and 500 mg D-galactosamine/ kg). Similarly, recombinant BPI binds LPS and inhibits TNF formation *in vitro*. Treatment comprised 5 mg BPI/kg i.v. for the BPI group, or 1 ml saline i.v. for the control group.

The results of the investigation of BPI efficacy in rats with either (a) hemorrhagic shock or (b) endotoxic shock show that (a) in rats with hemorrhagic shock, the mortality was decreased from 5/10 (50% control group) to 2/10 (20% BPI group) at 48 hours; (b) in rats with endotoxic shock, the 5-day mortality was significantly reduced ($p = 0.055$) by BPI treatment to 43%, as compared to 83% in the control group. Plasma LPS levels were at least partially neutralized at two hours (5.9 ± 4.1 vs 10.8 ± 4.1 ng/ml). Cytokine formation was concomitantly reduced in the BPI group as measured by plasma TNF levels at two hours (3.9 ± 2.9 vs 10.3 ± 6.3 ng/ml). Liver transaminases (GOT and GPT, whose elevation indicates hepatic dysfunction) and bilirubin still increased at eight hours; however, the increase was less with BPI. These data demonstrate that BPI has utility as a therapeutic agent against endotoxin-related disorders in hemorrhagic as well as endotoxic shock.

Example 14: Protection against LPS challenge by intrapulmonary delivery of RENPs

Anesthetized male CD-1 mice were treated intra-nasally with 1 or 10 μg of either BPI or $L_{1-197(I43->V)}B_{200-456(N206->D)}$ in 50 μl. Control animals received 50 μl of saline for injection. After 20 minutes, animals were re-anesthetized, and challenged with 10 ng of E. coli 055:B5 LPS. One hour after endotoxin challenge, mice were re-anesthetized, and 0.7 ml of saline containing 1% human serum albumin was added to the lungs via the trachea. The lungs were gently kneaded. A 0.5 ml volume of BAL (bronchoalveolar lavage) fluid was aspirated, cells were pelleted by centrifugation, and the BAL sample was sorted at -70°C. The TNF-alpha level in the BAL fluid was determined by ELISA (results shown in Figure 19).

Figure 19 shows that endotoxin-neutralizing proteins such as BPI and $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103) can also neutralize endotoxin-mediated TNF release in the lung. These results indicate that these proteins are effective when delivered directly into the lung and thus may be useful for treatment of pneumonias and other endotoxin-related disorders of the lung, such as ARDS.

Example 15. Construction of $L_{1-197}B_{200-456}$

cDNA encoding $L_{1-197}B_{200-456}$ was constructed by creating a unique ClaI site at the junction between the nucleotide sequence coding for Ile_{197}-Asp_{198} residues (ATA-GAT -> ATC-GAT). For $L_{1-197}B_{200-456}$, a 0.7 kb NheI/ClaI DNA fragment (encoding amino acids 1-197) derived from the 5′ sequence of LBP and a 0.8 kb ClaI/XhoI fragment (encoding amino acids 200-456) derived from the 3′ sequence of BPI were generated by PCR. The chimeric cDNAs were spliced together by cloning the

fragments into pSE, a mammalian vector. The cDNAs for BPI, LBP and $L_{1-197}B_{200-456}$ were transfected into Chinese hamster ovary cells (strain DUXB11) using lipofectin. The resulting transformed cells were selected, and expression was amplified with methotrexate. Cell culture supernatants were screened for reactivity by ELISA. Recombinant BPI, LBP, and $L_{1-197}B_{200-456}$ were purified as described above.

Example 16. Pharmokinetics of $L_{1-197}B_{200-456}$

Data for pharmacokinetic analysis were collected from healthy CD-1 mice given a single bolus injection (5 mg/kg) of recombinant protein at time=0. Blood was collected from three mice for each collection time point by retroorbital puncture at timepoints over three hours. Blood samples anticoagulated in EDTA were assayed by a double antibody sandwich ELISA for the presence of BPI, LBP or $L_{1-197}B_{200-456}$). Pharmacokinetic analysis was performed using a non-compartmental analysis (PharmK pharmacokinetic software, SoftRes, Inc.).

Comparison of BPI and LBP shows that BPI was cleared rapidly with a clearance rate of 13.0 ml/minute (Table 12). LBP had the longest half life, with a clearance rate of 0.042 ml/min. Compared to BPI, LBP was cleared 310 times more slowly. $L_{1-197}B_{200-456}$ had an intermediate half life (Clearance rate = 0.175 ml/min), being cleared 74 times more slowly than BPI.

Table 12: Clearance rate of $L_{1-197}B_{200-456}$

	CL (ml/min)	(vs. BPI)
BPI	13.000	-
LBP	0.042	(310 fold)
$L_{1-197}B_{200-456}$	0.175	(74 fold)

Example 17. LPS protection by $L_{1-197}B_{200-456}$

Female CD-1 mice (n=10) were injected in the lateral tail vein with 35 mg/kg S. abortus equi LPS (Sigma, St. Louis, MO) at time=0. Recombinant protein (5 mg/kg) was then administered intravenously into the opposite lateral tail vein immediately following (t=0) endotoxin challenge. Survival was monitored at 24, 48 and 72 hours post-challenge. Control animals received 0.1 ml saline instead of recombinant protein. The p values were determined by Fisher's exact test.

The results are shown in Figure 20. BPI and $L_{1-197}B_{200-456}$ provided 90% to 100% survival, respectively, at the 72 hour end point. No further mortality was noted at seven days post-challenge. The untreated control group had a survival rate of 20%. The survival rates of the treated groups were statistically significant compared to the control group ($p<.001$ for the $L_{1-197}B_{200-456}$ group and $p=.003$ for the BPI group determined by Fisher's exact test). These results indicate that $L_{1-197}B_{200-456}$ is as effective as BPI in this endotoxin challenge model in vivo.

Example 18. Protection Against Endotoxin Challenge in Mice

The ability of the recombinant, endotoxin-neutralizing proteins $B_{(1-41)}L_{(1-199)}B_{(1-456)}$, $L_{(1-164)}B_{(200-456)}$, $B_{(1-175)}B_{(200-456)}$, $B_{(1-236)}$, and $B_{(1-190)}$ to protect mice against endotoxin challenge was carried out as described in Example 17 above. Protection by these proteins was compared to the protection provided by BPI or saline. The results of these studies are shown in Table 13.

TABLE 13

			Number of Survivors/10 at Time (hours)							
	Compound	Lot #	0	12	18	24	36	48	60	72
Group 1	native BPI	149724	10	10	10	10	10	9	9	9
Group 2	$B_{(1-41)}L_{(1-199)}B_{(1-456)}$	162303	10	10	10	10	9	9	8	8
Group 3	$L_{(1-164)}B_{(200-456)}$	164325	10	10	9	9	8	8	7	7
Group 4	$L_{(1-175)}B_{(200-456)}$	164326	10	10	10	10	10	10	10	10
Group 5	$B_{(1-236)}$	159695	8	7	5	4	1	0	0	0
Group 6	$B_{(1-190)}$	159699	10	9	8	6	6	6	5	5
Group 7	Saline		10	8	7	6	4	3	3	3

Each animal received 35 mg/kg LPS in 0.1 ml, followed immediately by 5 mg/kg of the indicated compound in 0.1 ml. Survival was monitored at each time point indicated.

Example 19: Detection of a Gram-negative infection in a patient

A blood sample of about 1 ml to 5 ml is drawn from a patient suspected of having a Gram-negative infection. The blood sample is treated with citrate anti-coagulant and plasma is separated from the blood cells by centrifugation. The plasma is then diluted in a series of 10-fold dilutions in assay buffer (pyrogen-free TBS + 1 mg/ml low endotoxin BSA, and 0.05% Tween-20). The diluted plasma samples are then mixed with a known amount of biotinylated RENP. A series of control samples containing known amounts of biotinylated RENP in assay

buffer is included in the assay as quantitative and negative controls.

The test and control samples are then applied to the wells of a microtiter plate having bound LPS. The LPS-bound microtiter wells are prepared by incubation with 1 or 4 μg of S. minnesota R595 Re LPS (LIST Biological Labs, Inc., #304) in 50 mM borate pH 9.5-9.8 + 20-25 mM EDTA overnight at 37°C. Blank, non-LPS coated wells are included on each plate as controls for non-specific binding. The plates are then washed extensively under running distilled deionized water, then dried at 37°C. The assay wells are subsequently blocked for 60 minutes at 37°C with 1-2% very low endotoxin BSA (Sigma, St. Louis, MO) prepared in pyrogen-free Tris-buffered saline (50 mM Tris pH 7.4 + 150 mM NaCl).

The test and control samples are incubated for a time sufficient for binding of the RENP in the samples to the LPS bound to the microtiter wells, generally about 2-3 hours at 37°C in a total volume of 100 μl/well. After incubation, the wells are washed four times with assay buffer, and the plates are developed with streptavidin conjugated to alkaline phosphatase followed by 100 μl of PNP substrate solution freshly prepared from two 5 mg tablets dissolved in 10 ml substrate buffer. Substrate buffer is prepared with 24.5 mg $MgCl_2$, 48 ml diethanolamine, brought up to 400 ml, pH adjusted to 9.8 and volume brought up to 500 ml. Absorbances are read at 405 nm on a microplate reader.

If the level of biotinylated RENP bound to the wells of the test sample is significantly less than the level of biotinylated RENP bound to the negative control sample, then the patient has endotoxin circulating in the bloodstream which is generally associated with a Gram-negative infection. Moreover, the level of RENP binding in the test sample is compared to the levels of

RENP binding in the quantitative controls, ach of which are representative of varying degrees of severity of Gram-negative infection in a patient. The level of binding of the test sample is thus compared to the levels of binding of the quantitative samples to determine a degree of severity of infection.

Example 20: Detection of a Gram-negative infection *in vivo*

RENP is detectably labeled with ^{125}I using methods well known in the art. Approximately 100 μg of an ^{125}I-labeled RENP is injected intravenously into a patient suspected of having a Gram-negative infection in an organ, e.g., the liver. After allowing a time sufficient for circulation of the ^{125}I-labeled RENP to the suspected site of infection, the abdomen of the patient is fluoroscoped or X-rayed 2 to 3 times so as to include various perspectives. The X-ray is then examined to identify sites of binding of the RENP by virtue of an abnormally darkened section on the X-ray. Upon identification of the site of infection, the clinician designs an appropriate therapeutic regimen.

Following procedures similar to those described above, other recombinant, LPS-binding proteins can be produced and used in diagnostic methods and methods of treatment according to the invention.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

CLAIMS - 87 -

1. A method of detecting a site of Gram-negative bacterial infection in a subject, said method comprising the steps of:

injecting into the patient's circulatory system an injectable formulation comprising an effective amount of a recombinant endotoxin-neutralizing polypeptide attached to a detectable label, wherein the polypeptide is characterized by (i) selective and specific binding to lipopolysaccharide and (ii) endotoxin-neutralizing activity, with the proviso that the amino acid sequence of the polypeptide is not identical to the amino acid sequences of BPI or LBP;

allowing the detectably labeled polypeptide sufficient time to circulate in the subject and bind to lipopolysaccharide in the patient; and

detecting a site of label binding in the patient, thereby detecting a site of Gram-negative bacterial infection.

2. The method of claim 1, wherein the polypeptide is covalently bound to a molecule which enhances the half-life of the polypeptide.

3. The method of claim 1, wherein the polypeptide contains an LPS binding domain of BPI, LBP, a BPI variant, or an LBP variant.

4. The method of claim 1, wherein the detectable label is a radionucleotide.

5. A method of detecting a Gram-negative bacterial infection in a subject, said method comprising the steps of:

obtaining a sample from a patient suspected of having a Gram-negative bacterial infection;

contacting said sample with a detectably labeled recombinant endotoxin-neutralizing polypeptide for a time sufficient for binding of the polypeptide to lipopolysaccharide in the sample; and

detecting formation of lipopolysaccharide-polypeptide complexes by detection of a detectable label bound to the polypeptide;

wherein detection of a level of detectable label in said sample significantly greater than a level of detectable label in a negative control sample is indicative of a Gram-negative bacterial infection in the subject.

6. The method of claim 5, wherein the polypeptide contains an LPS binding domain of BPI, LBP, a BPI variant, or an LBP variant.

7. The method of claim 5, wherein said detection is quantitative.

8. The method of claim 7, wherein said quantitative detection is correlated with an Gram-negative bacterial infection load.

9. A detectably lab led recombinant endotoxin-neutralizing polypeptide characterized by (i) selective and specific binding to lipopolysaccharide and (ii) endotoxin-neutralizing activity, with the proviso that the amino acid sequence of the polypeptide is not identical to the amino acid sequences of BPI or LBP.

10. A polypeptide according to claim 9, wherein the polypeptide contains an LPS binding domain of BPI, LBP, a BPI variant, or an LBP variant.

11. A detectably labeled polypeptide according to claim 9, wherein the polypeptide comprises a molecule which enhances the half-life of said polypeptide and is covalently bound to the polypeptide.

12. A detectably labeled polypeptide according to claim 11, wherein said molecule is an immunoglobulin fragment, a half-life enhancing porion of LBP, a half-life enhancing portion of an LBP variant, or polyethylene glycol.

13. A kit for detecting a site of Gram-negative bacterial infection in a subject, the kit comprising:

an injectable formulation comprising a detectably labeled recombinant endotoxin-neutralizing polypeptide characterized by (i) selective and specific binding to lipopolysaccharide and (ii) endotoxin-neutralizing activity, with the proviso that the amino acid sequence of the polypeptide is not identical to the amino acid sequences of BPI or LBP.

14. A recombinant endotoxin-neutralizing polypeptide characterized by (i) selective and specific binding to lipopolysaccharide and (ii) endotoxin-neutralizing activity, with the proviso that the amino acid sequence of the polypeptide is not identical to the amino acid sequences of BPI or LBP.

15. A recombinant endotoxin-neutralizing polypeptide according to claim 14, wherein said polypeptide is of the formula $L_{1-197}B_{200-456}$ or a corresponding protein which (a) functions to bind lipopolysaccharide and (b) neutralizes endotoxin.

16. A recombinant endotoxin-neutralizing polypeptide according to claim 14, wherein the polypeptide is BPI(S351->X), wherein X is any amino acid other than serine.

17. A recombinant endotoxin-neutralizing polypeptide according to claim 16, wherein X is alanine.

18. A recombinant endotoxin-neutralizing polypeptide according to claim 14, wherein the polypeptide contains the amino acid sequence of BPI having a cationic amino acid substituted with a neutral or anionic residue.

19. A recombinant endotoxin-neutralizing polypeptide according to claim 18, wherein the cationic amino acid is at BPI amino acid residue positions 27, 30, 33, 42, 44, 48, 59, 77, 86, 90, 96, 118, 127, 148, 150, 160, 161, 167, 169, 177, 185, or 198.

20. A recombinant endotoxin-neutralizing polypeptide according to claim 19, wherein the polypeptide contains neutral or anionic residues at BPI amino acid residue positions 27, 30, 33, 42, 44, 48, and 59.

21. A recombinant endotoxin-neutralizing polypeptide according to claim 19, wherein the polypeptide contains neutral or anionic residues at BPI amino acid residue positions 77, 86, 90, 96, 118, and 127.

22. A recombinant endotoxin-neutralizing polypeptide according to claim 19, wherein the polypeptide contains neutral or anionic residues at BPI amino acid residue positions 148, 150, 160, 161, 167, 169, 177, 185, and 198.

23. A recombinant endotoxin-neutralizing polypeptide according to claim 18, wherein the polypeptide contains neutral or anionic residues at BPI amino acid residue positions 27, 30, 33, 42, 44, 48, 59, 77, 86, 90, 96, 118, 127, 148, 150, 160, 161, 167, 169, 177, 185, and 198.

24. A recombinant endotoxin-neutralizing polypeptide according to claim 14, wherein the polypeptide contains the amino acid sequence of LBP having an amino acid substituted for an amino acid in a corresponding amino acid residue position of BPI.

25. A recombinant endotoxin-neutralizing polypeptide according to claim 24, wherein the amino acid substituted is at LBP amino acid residue positions 77, 86, 96, 118, 126, 147, 148, 158, 159, 161, 165, 167, 175, 183, or 196.

26. A recombinant endotoxin-neutralizing polypeptide according to claim 14, wherein the polypeptide contains the amino acid sequence of BPI having a cysteine residue substituted with an amino acid other than cysteine.

27. A recombinant endotoxin-neutralizing polypeptide according to claim 26, wherein said cysteine residue is at BPI amino acid residue position 132, 135, or 175.

28. A recombinant endotoxin-neutralizing polypeptide according to claim 26, wherein the cysteine residues of BPI at positions 132, 135, and 175 are substituted with an amino acid other than cysteine.

29. A recombinant endotoxin-neutralizing polypeptide according to claim 14, wherein the polypeptide comprises a molecule which enhances the half-life of said polypeptide and is covalently bound to the polypeptide.

30. A recombinant endotoxin-neutralizing polypeptide according to claim 29, wherein said polypeptide contains a lipopolysaccharide-binding domain of BPI, LBP, a BPI variant, or an LBP variant.

31. A recombinant endotoxin-neutralizing polypeptide according to claim 29, wherein said molecule is an immunoglobulin fragment, a half-life enhancing portion of LBP, a half-life enhancing portion of an LBP variant, or polyethylene glycol.

32. A recombinant endotoxin-neutralizing polypeptide according to claim 29, wherein the endotoxin-neutralizing polypeptide of (a) is a C-terminal fragment of BPI and the molecule of (b) is an N-terminal fragment of LBP.

33. A recombinant endotoxin-neutralizing polypeptide according to claim 32, wherein said C-terminal fragment of BPI is a fragment having an amino acid sequence contained in BPI amino acid residues 60-456.

34. A recombinant endotoxin-neutralizing polypeptide according to claim 33, wherein said C-terminal fragment of BPI is BPI amino acid residues 60-456, 136-456, 277-456, 300-456, 200-456, 136-361, 136-275, 200-275, or 200-361.

35. A recombinant endotoxin-neutralizing polypeptide according to claim 32, wherein said N-terminal fragment of LBP is a fragment having an amino acid sequence contained in LBP amino acid residues 1-175.

36. A recombinant endotoxin-neutralizing polypeptide according to claim 21, wherein said N-terminal fragment of LBP is LBP amino acid residues 1-59, 1-134, 1-164, 1-175, 1-274, 1-359, 1-134, or 1-197.

37. A recombinant endotoxin-n utralizing polypeptide of claim 18, wherein the polypeptide further comprises a C-terminal fragment of LBP.

38. A recombinant endotoxin-neutralizing polypeptide of claim 23, wherein the C-terminal fragment of LBP is LBP amino acid residues 360-456 or 274-456.

39. An isolated DNA molecule encoding a recombinant endotoxin binding polypeptide according to claim 14.

40. A vector comprising the DNA of claim 39.

41. A transformed host cell comprising the DNA of claim 39.

42. A method for producing a recombinant endotoxin-neutralizing polypeptide according to claim 14, said method comprising the steps of:

culturing a transformed host cell comprising DNA encoding a recombinant endotoxin binding polypeptide according to claim 14, said DNA being operably linked to a promoter for expression of the polypeptide encoded by the DNA, said culturing being under conditions allowing expression of said polypeptide; and

isolating the recombinant endotoxin binding polypeptide produced.

43. A pharmaceutical composition comprising:

a therapeutically effective amount of a recombinant endotoxin-neutralizing polypeptide according to claim 14; and

a pharmaceutically acceptable carrier.

44. A method f treating a subject suffering from an endotoxin-related disorder, said method comprising:

administering to a subject having an endotoxin-related disorder a therapeutically effective amount of a recombinant endotoxin-neutralizing polypeptide according to claim 14, wherein LPS-mediated stimulation of neutrophils and mononuclear cells is inhibited.

45. A method of preventing an endotoxin-related disorder in a subject, said method comprising:

administering to a subject a prophylactically effective amount of a recombinant endotoxin-neutralizing polypeptide according to claim 14, wherein the endotoxin-related disorder is prevented.

FIGURE 1A

FIGURE 1A
FIGURE 1B
FIGURE 1C
FIGURE 1D

-25

Human LBP-a (as reported by Schumann et al.) m
Human LBP-b (correct sequence) m

-20 -10

g a l a h a l p s i l l a l l l t s t p e a l g
g a l a h a l p s i l l a l l l t s t p e a l g

10 20

Human LBP-a A N P G L V A R I T D K G L Q Y A A Q E G L L A L
Human LBP-b A N P G L V A R I T D K G L Q Y A A Q E G L L A L

30 40 50

Q S E L L R I T L P D F T G D L R I P H V G R G R
Q S E L L R I T L P D F T G D L R I P H V G R G R

60 70

Human LBP-a Y E F H S L N I H S C E L L H S A L R P V P G Q G
Human LBP-b Y E F H S L N I H S C E L L H S A L R P V P G Q G

FIGURE 1B

```
                                80                                  90                                  100
                L  S  L  S  I  S  D  S  S  I  R  V  Q  G  R  W  K  V  R  K  S  F  E  K  I
                L  S  L  S  I  S  D  S  S  I  R  V  Q  G  R  W  K  V  R  K  S  F  F  K  I

                                          110                                 120
Human  LBP-a    Q  G  S  F  D  V  S  V  K  G  I  S  I  S  V  N  L  L  L  G  S  E  S  S
Human  LBP-b    Q  G  S  F  D  V  S  V  K  G  I  S  I  S  V  N  L  L  L  G  S  E  S  S

                          130                                 140
                G  R  P  T  G  C  Y  L  S  C  S  S  D  I  A  D  V  E  V  D  M  S  G  D
                G  R  P  T  V  T  A  S  S  C  S  S  D  I  A  D  V  E  V  D  M  S  G  D
                         *  *  *  *

                150                                 160                                 170
Human  LBP-a    S  G  W  L  L  N  L  F  H  N  Q  I  E  S  K  F  Q  K  V  L  E  S  R  I  C
Human  LBP-b    F  G  W  L  L  N  L  F  H  N  Q  I  E  S  K  F  Q  K  V  L  E  S  R  I  C

                                180                                 190                                 25K/30K
                E  M  I  Q  K  S  V  S  S  D  L  Q  P  Y  L  Q  T  L  P  V  T  T  E  I  D
                E  M  I  Q  K  S  V  S  S  D  L  Q  P  Y  L  Q  T  L  P  V  T  T  E  I  D
```

FIGURE 1C

```
                  *200                 210                 220
Human  LBP-a  S F A D I D Y S L V E A P R A T A Q M L E V M F K
Human  LBP-b  S F A D I D Y S L V E A P R A T A Q M L E V M F K

                  230                 240
              G E I F H R N H R S P V T L L A A A - - - - E E H
              G E I F H R N H R S P V T L L A A V M S L P E E H
                                        *   *   *   *   *

                  250                 260                 270
Human  LBP-a  N K M V Y F A I S D Y V F N T A S L V Y H E E G Y
Human  LBP-b  N K M V Y F A I S D Y V F N T A S L V Y H E E G Y

                  280                 290
              L N F S I T D D M I P P D S N I R L T T K S F R P
              L N F S I T D D M I P P D S N I R L T T K S F R P

                  300                 310                 320
Human  LBP-a  F V P R L A R L Y P N M N L E L Q G S V P S A P L
Human  LBP-b  F V P R L A R L Y P N M N L E L Q G S V P S A P L
```

FIGURE 1D

		330										340														
		L	N	F	S	P	G	N	L	S	V	D	P	Y	M	E	I	D	A	F	V	L	L	P	S	S
		L	N	F	S	P	G	N	L	S	V	D	P	Y	M	E	I	D	A	F	V	L	L	P	S	S
		350								360								370								
Human	LBP-a	S	K	E	P	V	F	R	L	S	V	A	T	N	V	S	A	T	L	T	F	N	T	S	K	I
Human	LBP-b	S	K	E	P	V	F	R	L	S	V	A	T	N	V	S	A	T	L	T	F	N	T	S	K	I
		380										390														
		T	G	F	L	K	P	G	K	V	K	V	E	L	K	E	S	K	V	G	L	F	N	A	L	L
		T	G	F	L	K	P	G	K	V	K	V	E	L	K	E	S	K	V	G	L	F	N	A	E	L
		400								410								420								
Human	LBP-a	L	E	A	L	L	N	Y	Y	I	L	N	T	L	Y	P	K	F	N	D	K	L	A	E	G	F
Human	LBP-b	L	E	A	L	L	N	Y	Y	I	L	N	T	F	Y	P	K	F	N	D	K	L	A	E	G	F
		430										440														
		P	L	P	L	L	K	R	V	Q	L	Y	D	L	G	L	Q	I	H	K	D	F	L	F	L	G
		P	L	P	L	L	K	R	V	Q	L	Y	D	L	G	L	Q	I	H	K	D	F	L	F	L	G
		450							456																	
Human	LBP-a	A	N	V	Q	Y	M	R	V																	
Human	LBP-b	A	N	V	Q	Y	M	R	V																	

Figure 2

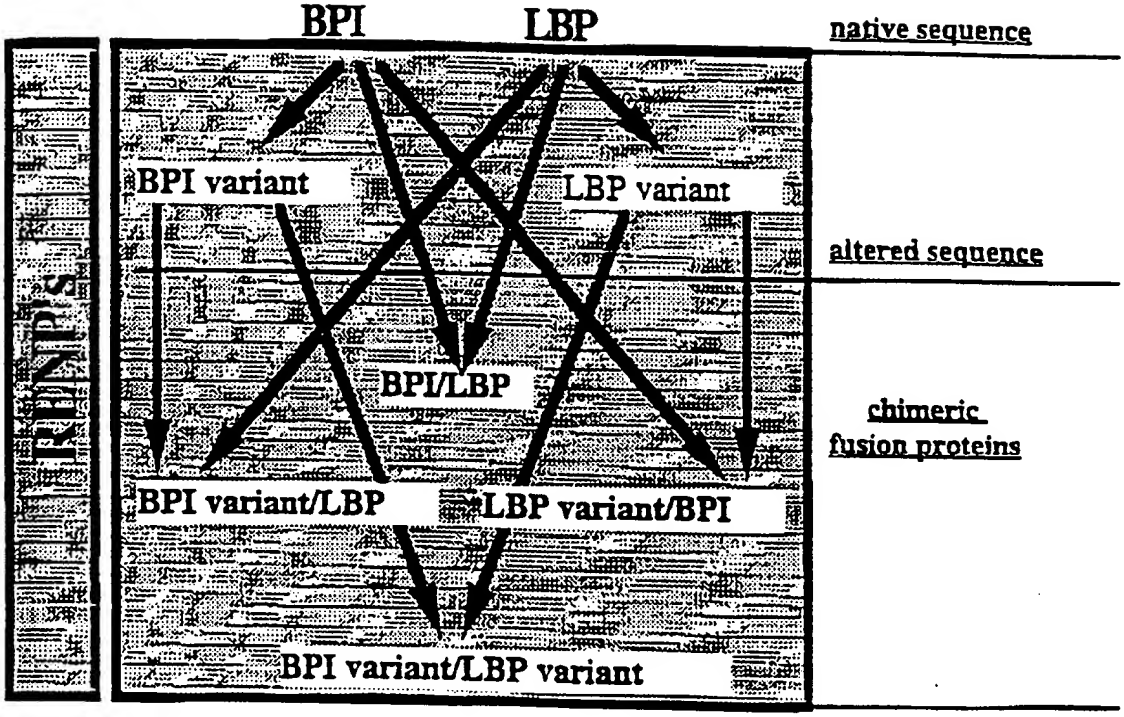

BPI
LBP
native sequence
BPI variant
LBP variant
altered sequence
BPI/LBP
chimeric
fusion proteins
BPI variant/LBP
LBP variant/BPI
BPI variant/LBP variant
BPI/LBP

FIGURE 3A

FIGURE 3A
FIGURE 3B
FIGURE 3C
FIGURE 3D

BPI cDNA

```
   1  CAG GCC TTG AGG TTT TGG CAG CTC TGG AGG ATG AGA GAG AAC ATG GCC   48
   1                                        Met Arg Glu Asn Met Ala    6

  49  AGG GGC CCT TGC AAC GCG CCG AGA TGG GTG TCC CTG ATG GTG CTC GTC   96
   7  Arg Gly Pro Cys Asn Ala Pro Arg Trp Val Ser Leu Met Val Leu Val   22

  97  GCC ATA GGC ACC GCC GTG ACA GCG GCC GTC AAC CCT GGC GTC GTG GTC  144
  23  Ala Ile Gly Thr Ala Val Thr Ala Ala Val Asn Pro Gly Val Val Val   38

 145  AGG ATC TCC CAG AAG GGC CTG GAC TAC GCC AGC CAG CAG GGG ACG GCC  192
  39  Arg Ile Ser Gln Lys Gly Leu Asp Tyr Ala Ser Gln Gln Gly Thr Ala   54

 193  GCT CTG CAG AAG GAG CTG AAG AGG ATC AAG ATT CCT GAC TAC TCA GAC  240
  55  Ala Leu Gln Lys Glu Leu Lys Arg Ile Lys Ile Pro Asp Tyr Ser Asp   70

 241  AGC TTT AAG ATC AAG CAT CTT GGG AAG GGG CAT TAT AGC TTC TAC AGC  288
  71  Ser Phe Lys Ile Lys His Leu Gly Lys Gly His Tyr Ser Phe Tyr Ser   86

 289  ATG GAC ATC CGT GAA TTC CAG CTT CCC AGT TCC CAG ATA AGC ATG GTG  336
  87  Met Asp Ile Arg Glu Phe Gln Leu Pro Ser Ser Gln Ile Ser Met Val  102
```

FIGURE 3B

```
337  CCC AAT GTG GGC CTT AAG TTC TCC ATC AGC AAC GCC AAT ATC AAG ATC   384
103  Pro Asn Val Gly Leu Lys Phe Ser Ile Ser Asn Ala Asn Ile Lys Ile   118

385  AGC GGG AAA TGG AAG GCA CAA AAG AGA TTC TTA AAA ATG AGC GGC AAT   432
119  Ser Gly Lys Trp Lys Ala Gln Lys Arg Phe Leu Lys Met Ser Gly Asn   134

433  TTT GAC CTG AGC ATA GAA GGC ATG TCC ATT TCG GCT GAT CTG AAG CTG   480
135  Phe Asp Leu Ser Ile Glu Gly Met Ser Ile Ser Ala Asp Leu Lys Leu   150

481  GGC AGT AAC CCC ACG TCA GGC AAG CCC ACC ATC ACC TGC TCC AGC TGC   528
151  Gly Ser Asn Pro Thr Ser Gly Lys Pro Thr Ile Thr Cys Ser Ser Cys   166

529  AGC AGC CAC ATC AAC AGT GTC CAC GTG CAC ATC TCA AAG AGC AAA GTC   576
167  Ser Ser His Ile Asn Ser Val His Val His Ile Ser Lys Ser Lys Val   182

577  GGG TGG CTG ATC CAA CTC TTC CAC AAA AAA ATT GAG TCT GCG CTT CGA   624
183  Gly Trp Leu Ile Gln Leu Phe His Lys Lys Ile Glu Ser Ala Leu Arg   198

625  AAC AAG ATG AAC AGC CAG GTC TGC GAG AAA GTG ACC AAT TCT GTA TCC   672
199  Asn Lys Met Asn Ser Gln Val Cys Glu Lys Val Thr Asn Ser Val Ser   214

673  TCC AAG CTG CAA CCT TAT TTC CAG ACT CTG CCA GTA ATG ACC AAA ATA   720
215  Ser Lys Leu Gln Pro Tyr Phe Gln Thr Leu Pro Val Met Thr Lys Ile   230

721  GAT TCT GTG GCT GGA ATC AAC TAT GGT CTG GTG GCA CCT CCA GCA ACC   768
231  Asp Ser Val Ala Gly Ile Asn Tyr Gly Leu Val Ala Pro Pro Ala Thr   246
```

FIGURE 3C

```
 769  ACG GCT GAG ACC CTG GAT GTA CAG ATG AAG GGG GAG TTT TAC AGT GAG    816
 247  Thr Ala Glu Thr Leu Asp Val Gln Met Lys Gly Glu Phe Tyr Ser Glu    262

 817  AAC CAC CAC AAT CCA CCT CCC TTT GCT CCA CCA GTG ATG GAG TTT CCC    864
 263  Asn His His Asn Pro Pro Pro Phe Ala Pro Pro Val Met Glu Phe Pro    278

 865  GCT GCC CAT GAC CGC ATG GTA TAC CTG GGC CTC TCA GAC TAC TTC TTC    912
 279  Ala Ala His Asp Arg Met Val Tyr Leu Gly Leu Ser Asp Tyr Phe Phe    294

 913  AAC ACA GCC GGG CTT GTA TAC CAA GAG GCT GGG GTC TTG AAG ATG ACC    960
 295  Asn Thr Ala Gly Leu Val Tyr Gln Glu Ala Gly Val Leu Lys Met Thr    310

 961  CTT AGA GAT GAC ATG ATT CCA AAG GAG TCC AAA TTT CGA CTG ACA ACC   1008
 311  Leu Arg Asp Asp Met Ile Pro Lys Glu Ser Lys Phe Arg Leu Thr Thr    326

1009  AAG TTC TTT GGA ACC TTC CTA CCT GAG GTG GCC AAG AAG TTT CCC AAC   1056
 327  Lys Phe Phe Gly Thr Phe Leu Pro Glu Val Ala Lys Lys Phe Pro Asn    342

1057  ATG AAG ATA CAG ATC CAT GTC TCA GCC TCC ACC CCG CCA CAC CTG TCT   1104
 343  Met Lys Ile Gln Ile His Val Ser Ala Ser Thr Pro Pro His Leu Ser    358

1105  GTG CAG CCC ACC GGC CTT ACC TTC TAC CCT GCC GTG GAT GTC CAG GCC   1152
 359  Val Gln Pro Thr Gly Leu Thr Phe Tyr Pro Ala Val Asp Val Gln Ala    374

1153  CTT GCC GTC CTC CCC AAC TCC TCC CTG GCT TCC CTC TTC CTG ATT GGC   1200
 375  Leu Ala Val Leu Pro Asn Ser Ser Leu Ala Ser Leu Phe Leu Ile Gly    390

1201  ATG CAC ACA ACT GGT TCC ATG GAG GTC AGC GCC GAG TCC AAC AGG CTT   1248
 391  Met His Thr Thr Gly Ser Met Glu Val Ser Ala Glu Ser Asn Arg Leu    406
```

FIGURE 3D

```
1249  GTT GGA GAG CTC AAG CTG GAT AGG CTG CTC CTG GAA CTG AAG CAC TCA   1296
 407  Val Gly Glu Leu Lys Leu Asp Arg Leu Leu Leu Glu Leu Lys His Ser    422

1297  AAT ATT GGC CCC TTC CCG GTT GAA TTG CTG CAG GAT ATC ATG AAC TAC   1344
 423  Asn Ile Gly Pro Phe Pro Val Glu Leu Leu Gln Asp Ile Met Asn Tyr    438

1345  ATT GTA CCC ATT CTT GTG CTG CCC AGG GTT AAC GAG AAA CTA CAG AAA   1392
 439  Ile Val Pro Ile Leu Val Leu Pro Arg Val Asn Glu Lys Leu Gln Lys    454

1393  GGC TTC CCT CTC CCG ACG CCG GCC AGA GTC CAG CTC TAC AAC GTA GTG   1440
 455  Gly Phe Pro Leu Pro Thr Pro Ala Arg Val Gln Leu Tyr Asn Val Val    470

1441  CTT CAG CCT CAC CAG AAC TTC CTG CTG TTC GGT GCA GAC GTT GTC TAT   1488
 471  Leu Gln Pro His Gln Asn Phe Leu Leu Phe Gly Ala Asp Val Val Tyr    486

1489  AAA TGA AGG CAC CAG GGG TGC CGG GGG CTG TCA GCC GCA CCT GTT CCT   1536
 487  Lys ***                                                          488

1537  GAT GGG CTG TGG GGC ACC GGC TGC CTT TCC CCA GGG AAT CCT CTC CAG   1584

1585  ATC TTA ACC AAG AGC CCC TTG CAA ACT TCT TCG ACT CAG ATT CAG AAA   1632

1633  TGA TCT AAA CAC GAG GAA ACA TTA TTC ATT GGA AAA GTG CAT GGT GTG   1680

1681  TAT TTT AGG GAT TAT GAG CTT CTT TCA AGG GCT AAG GCT GCA GAG ATA   1728

1729  TTT CCT CCA GGA ATC GTG TTT CAA TTG TAA CCA AGA AAT TTC CAT TTG   1776

1777  TGC TTC ATG AAA AAA AAC TTC TGG TTT TTT TCA TGT G                 1813
```

FIGURE 4A

FIGURE 4A
FIGURE 4B
FIGURE 4C

Human LBP Expression clone

```
   1  GCT AGC CCA CTG CAC TGG GAA TCT AGG ATG GGG GCC TTG GCG AGA GCC    48
   1   NheI                                Met Gly Ala Leu Ala Arg Ala     7

  49  CTG CCG TCC ATA CTG CTG GCA TTG CTG CTT ACG TCC ACC CCA GAG GCT    96
   8  Leu Pro Ser Ile Leu Leu Ala Leu Leu Leu Thr Ser Thr Pro Glu Ala    23

  97  CTG GGT GCC AAC CCC GGC TTG GTC GCC AGG ATC ACC GAC AAG GGA CTG   144
  24  Leu Gly Ala Asn Pro Gly Leu Val Ala Arg Ile Thr Asp Lys Gly Leu    39

 145  CAG TAT GCG GCC CAG GAG GGG CTA TTG GCT CTG CAG AGT GAG CTG CTC   192
  40  Gln Tyr Ala Ala Gln Glu Gly Leu Leu Ala Leu Gln Ser Glu Leu Leu    55

 193  AGG ATC ACG CTG CCT GAC TTC ACC GGG GAC TTG AGG ATC CCC CAC GTC   240
  56  Arg Ile Thr Leu Pro Asp Phe Thr Gly Asp Leu Arg Ile Pro His Val    71

 241  GGC CGT GGG CGC TAT GAG TTC CAC AGC CTG AAC ATC CAC AGC TGT GAG   288
  72  Gly Arg Gly Arg Tyr Glu Phe His Ser Leu Asn Ile His Ser Cys Glu    87

 289  CTG CTT CAC TCT GCG CTG AGG CCT GTC CCT GGC CAG GGC CTG AGT CTC   336
  88  Leu Leu His Ser Ala Leu Arg Pro Val Pro Gly Gln Gly Leu Ser Leu   103

 337  AGC ATC TCC GAC TCC TCC ATC CGG GTC CAG GGC AGG TGG AAG GTG CGC   384
 104  Ser Ile Ser Asp Ser Ser Ile Arg Val Gln Gly Arg Trp Lys Val Arg   119

 385  AAG TCA TTC TTC AAA CTA CAG GGC TCC TTT GAT GTC AGT GTC AAG GGC   432
 120  Lys Ser Phe Phe Lys Leu Gln Gly Ser Phe Asp Val Ser Val Lys Gly   135

 433  ATC AGC ATT TCG GTC AAC CTC CTG TTG GGC AGC GAG TCC TCC GGG AGG   480
 136  Ile Ser Ile Ser Val Asn Leu Leu Leu Gly Ser Glu Ser Ser Gly Arg   151
```

FIGURE 4B

```
481  CCC ACA GTT ACT GCC TCC AGC TGC AGC AGT GAC ATC GCT GAC GTG GAG   528
152  Pro Thr Val Thr Ala Ser Ser Cys Ser Ser Asp Ile Ala Asp Val Glu   167

529  GTG GAC ATG TCG GGA GAC TTC GGG TGG CTG TTG AAC CTC TTC CAC AAC   576
168  Val Asp Met Ser Gly Asp Phe Gly Trp Leu Leu Asn Leu Phe His Asn   183

577  CAG ATT GAG TCC AAG TTC CAG AAA GTA CTG GAG AGC AGG ATT TGC GAA   624
184  Gln Ile Glu Ser Lys Phe Gln Lys Val Leu Glu Ser Arg Ile Cys Glu   199

625  ATG ATC CAG AAA TCG GTG TCC TCC GAT CTA CAG CCT TAT CTC CAA ACT   672
200  Met Ile Gln Lys Ser Val Ser Ser Asp Leu Gln Pro Tyr Leu Gln Thr   215

673  CTG CCA GTT ACA ACA GAG ATT GAC AGT TTC GCC GAC ATT GAT TAT AGC   720
216  Leu Pro Val Thr Thr Glu Ile Asp Ser Phe Ala Asp Ile Asp Tyr Ser   231

721  TTA GTG GAA GCC CCT CGG GCA ACA GCC CAG ATG CTG GAG GTG ATG TTT   768
232  Leu Val Glu Ala Pro Arg Ala Thr Ala Gln Met Leu Glu Val Met Phe   247

769  AAG GGT GAA ATC TTT CAT CGT AAC CAC CGT TCT CCA GTT ACC CTC CTT   816
248  Lys Gly Glu Ile Phe His Arg Asn His Arg Ser Pro Val Thr Leu Leu   263

817  GCT GCA GTC ATG AGC CTT CCT GAG GAA CAC AAC AAA ATG GTC TAC TTT   864
264  Ala Ala Val Met Ser Leu Pro Glu Glu His Asn Lys Met Val Tyr Phe   279

865  GCC ATC TCG GAT TAT GTC TTC AAC ACG GCC AGC CTG GTT TAT CAT GAG   912
280  Ala Ile Ser Asp Tyr Val Phe Asn Thr Ala Ser Leu Val Tyr His Glu   295

913  GAA GGA TAT CTG AAC TTC TCC ATC ACA GAT GAC ATG ATA CCG CCT GAC   960
296  Glu Gly Tyr Leu Asn Phe Ser Ile Thr Asp Asp Met Ile Pro Pro Asp   311

961  TCT AAT ATC CGA CTG ACC ACC AAG TCC TTC CGA CCC TTC GTC CCA CGG  1008
312  Ser Asn Ile Arg Leu Thr Thr Lys Ser Phe Arg Pro Phe Val Pro Arg   327
```

FIGURE 4C

```
1009  TTA GCC AGG CTC TAC CCC AAC ATG AAC CTG GAA CTC CAG GGA TCA GTG   1056
 328  Leu Ala Arg Leu Tyr Pro Asn Met Asn Leu Glu Leu Gln Gly Ser Val    343

1057  CCC TCT GCT CCG CTC CTG AAC TTC AGC CCT GGG AAT CTG TCT GTG GAC   1104
 344  Pro Ser Ala Pro Leu Leu Asn Phe Ser Pro Gly Asn Leu Ser Val Asp    359

1105  CCC TAT ATG GAG ATA GAT GCC TTT GTG CTC CTG CCC AGC TCC AGC AAG   1152
 360  Pro Tyr Met Glu Ile Asp Ala Phe Val Leu Leu Pro Ser Ser Ser Lys    375

1153  GAG CCT GTC TTC CGG CTC AGT GTG GCC ACT AAT GTG TCC GCC ACC TTG   1200
 376  Glu Pro Val Phe Arg Leu Ser Val Ala Thr Asn Val Ser Ala Thr Leu    391

1201  ACC TTC AAT ACC AGC AAG ATC ACT GGG TTC CTG AAG CCA GGA AAG GTA   1248
 392  Thr Phe Asn Thr Ser Lys Ile Thr Gly Phe Leu Lys Pro Gly Lys Val    407

1249  AAA GTG GAA CTG AAA GAA TCC AAA GTT GGA CTA TTC AAT GCA GAG CTG   1296
 408  Lys Val Glu Leu Lys Glu Ser Lys Val Gly Leu Phe Asn Ala Glu Leu    423

1297  TTG GAA GCG CTC CTC AAC TAT TAC ATC CTT AAC ACC TTC TAC CCC AAG   1344
 424  Leu Glu Ala Leu Leu Asn Tyr Tyr Ile Leu Asn Thr Phe Tyr Pro Lys    439

1345  TTC AAT GAT AAG TTG GCC GAA GGC TTC CCC CTT CCT CTG CTG AAG CGT   1392
 440  Phe Asn Asp Lys Leu Ala Glu Gly Phe Pro Leu Pro Leu Leu Lys Arg    455

1393  GTT CAG CTC TAC GAC CTT GGG CTG CAG ATC CAT AAG GAC TTC CTG TTC   1440
 456  Val Gln Leu Tyr Asp Leu Gly Leu Gln Ile His Lys Asp Phe Leu Phe    471

1441  TTG GGT GCC AAT GTC CAA TAC ATG AGA GTT TGA GGA CAA GAA AGA TGA   1488
 472  Leu Gly Ala Asn Val Gln Tyr Met Arg Val ***                       482

1489  AGC TTG CTC GAG                                                  1500
           XhoI
```

FIGURE 5A

FIGURE 5A
FIGURE 5B
FIGURE 5C
FIGURE 5D
FIGURE 5E
FIGURE 5F

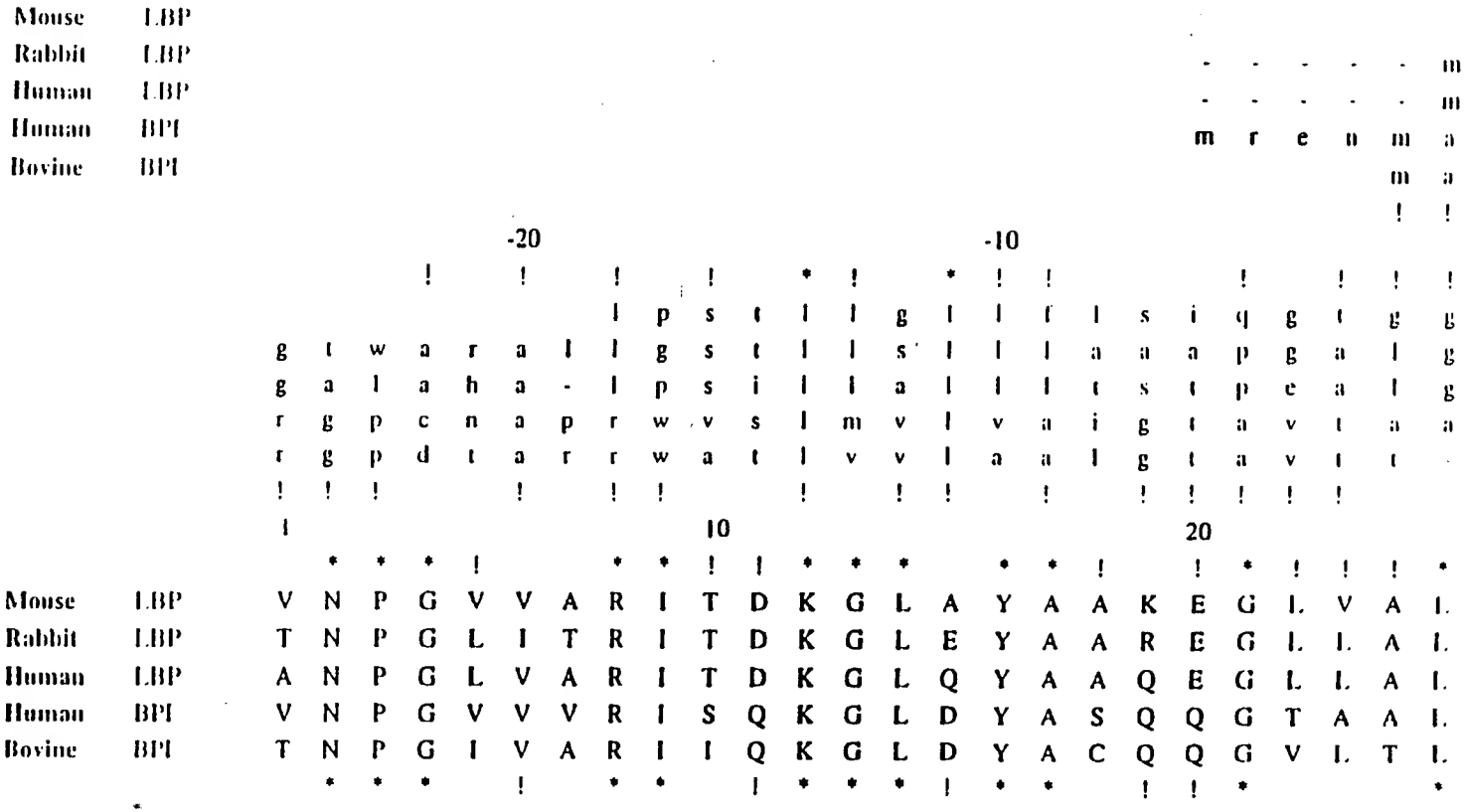


FIGURE 5B

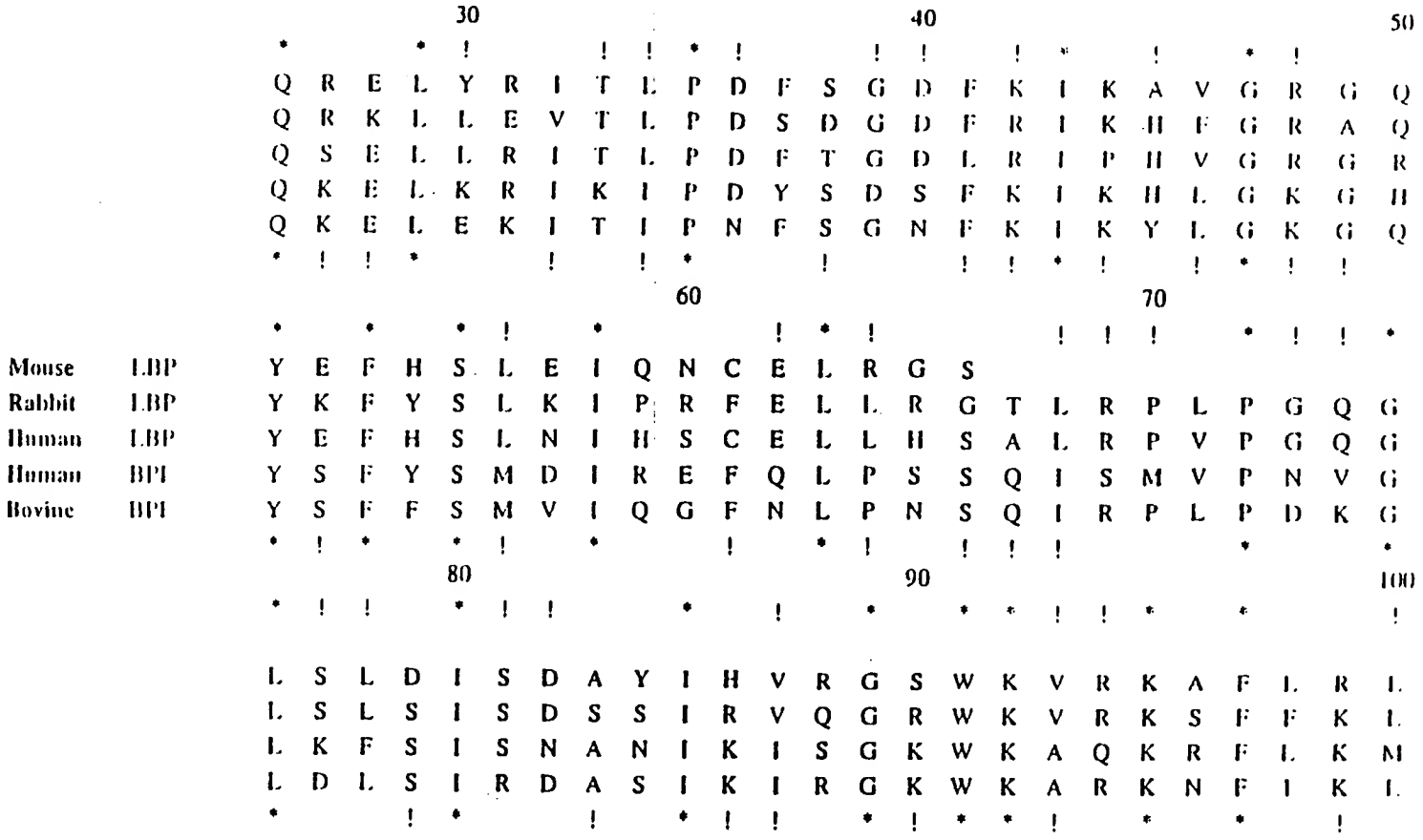

30
40
50
60
70
80
90
100
Mouse LBP
Rabbit LBP
Human LBP
Human BPI
Bovine BPI

FIGURE 5C

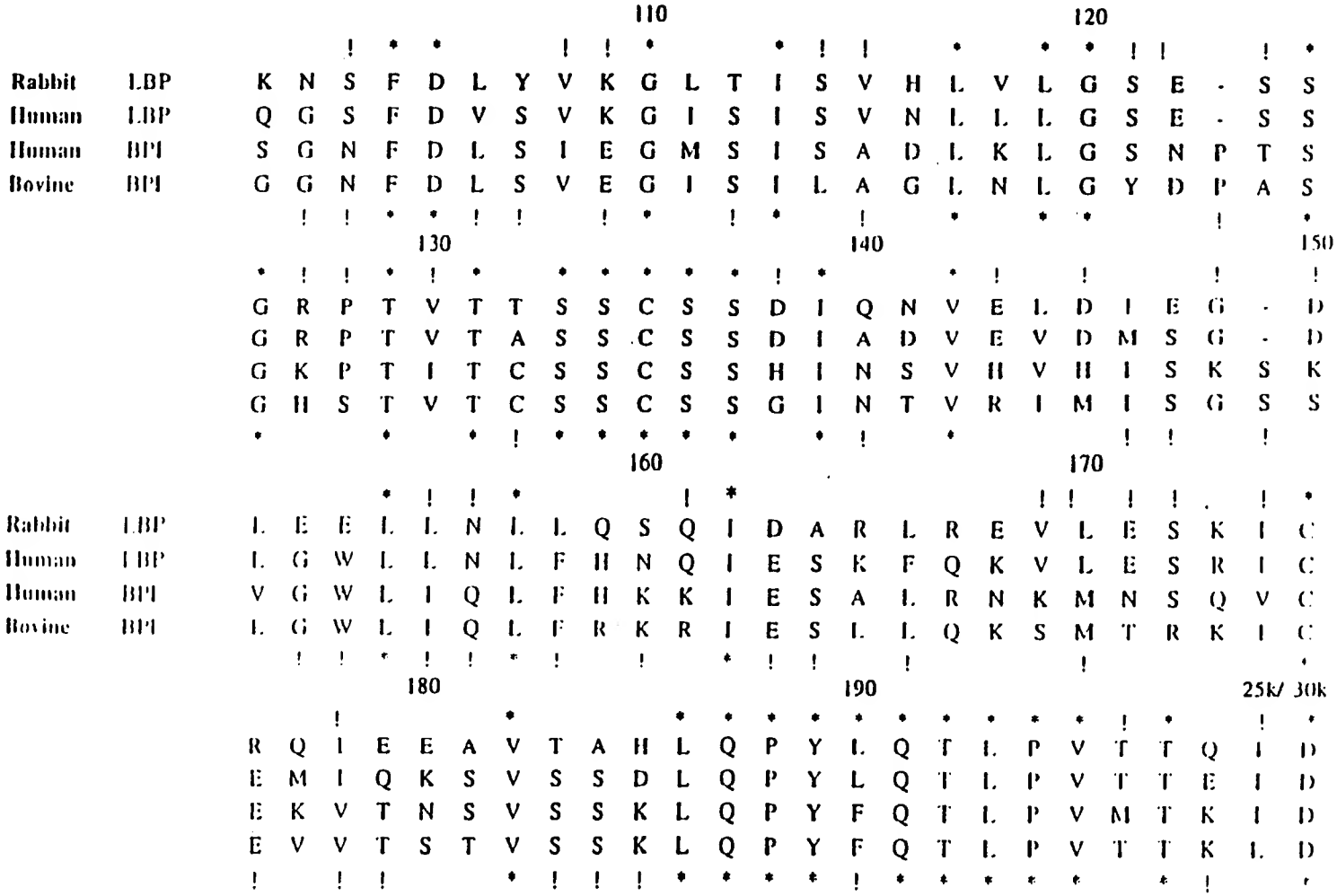

110
120
130
140
150
160
170
180
190
25k/ 30k
Rabbit LBP
Human LBP
Human BPI
Bovine BPI

FIGURE 5D

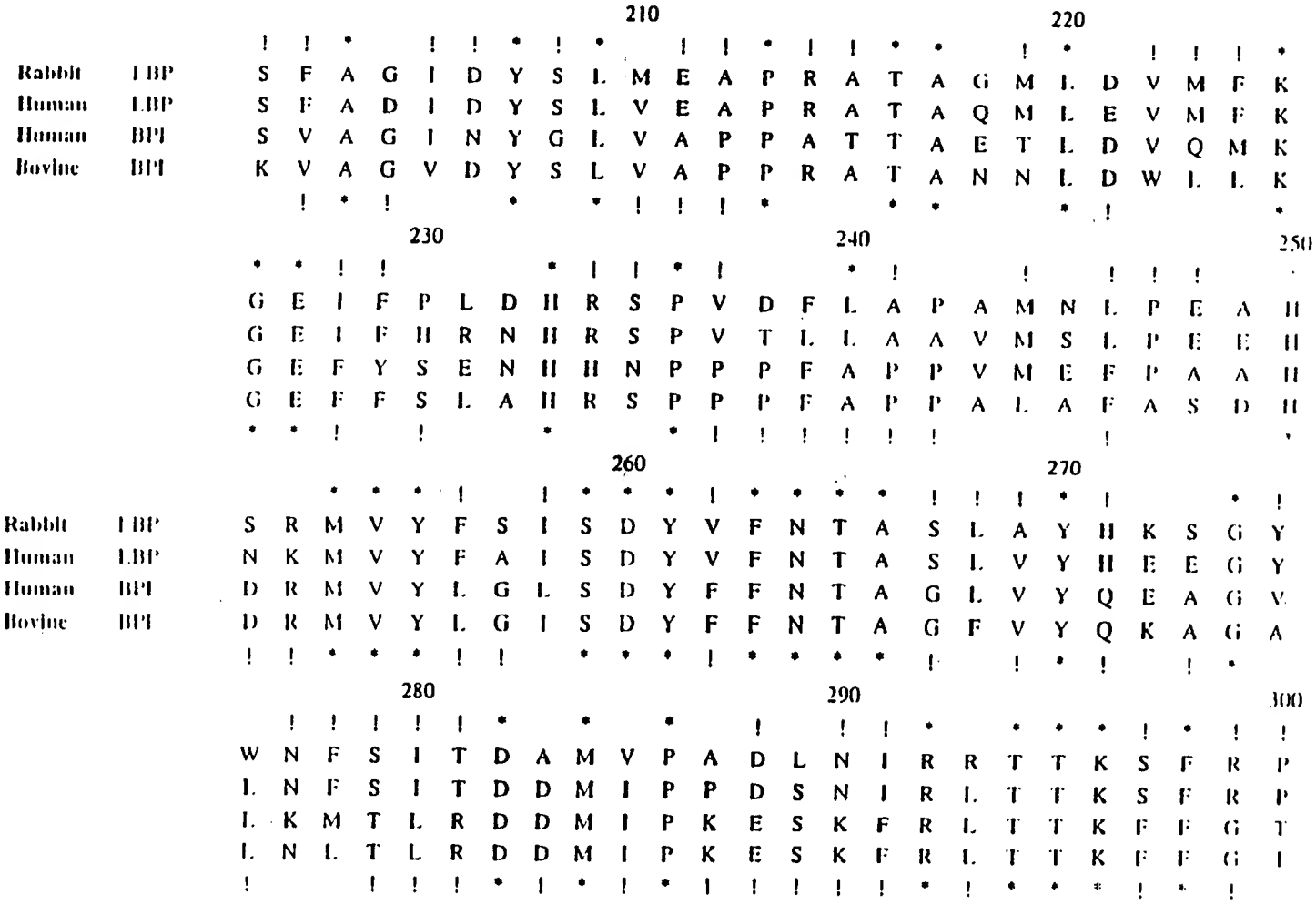

210
220
230
240
250
260
270
280
290
300
Rabbit LBP
Human LBP
Human BPI
Bovine BPI
Rabbit LBP
Human LBP
Human BPI
Bovine BPI

FIGURE 5E

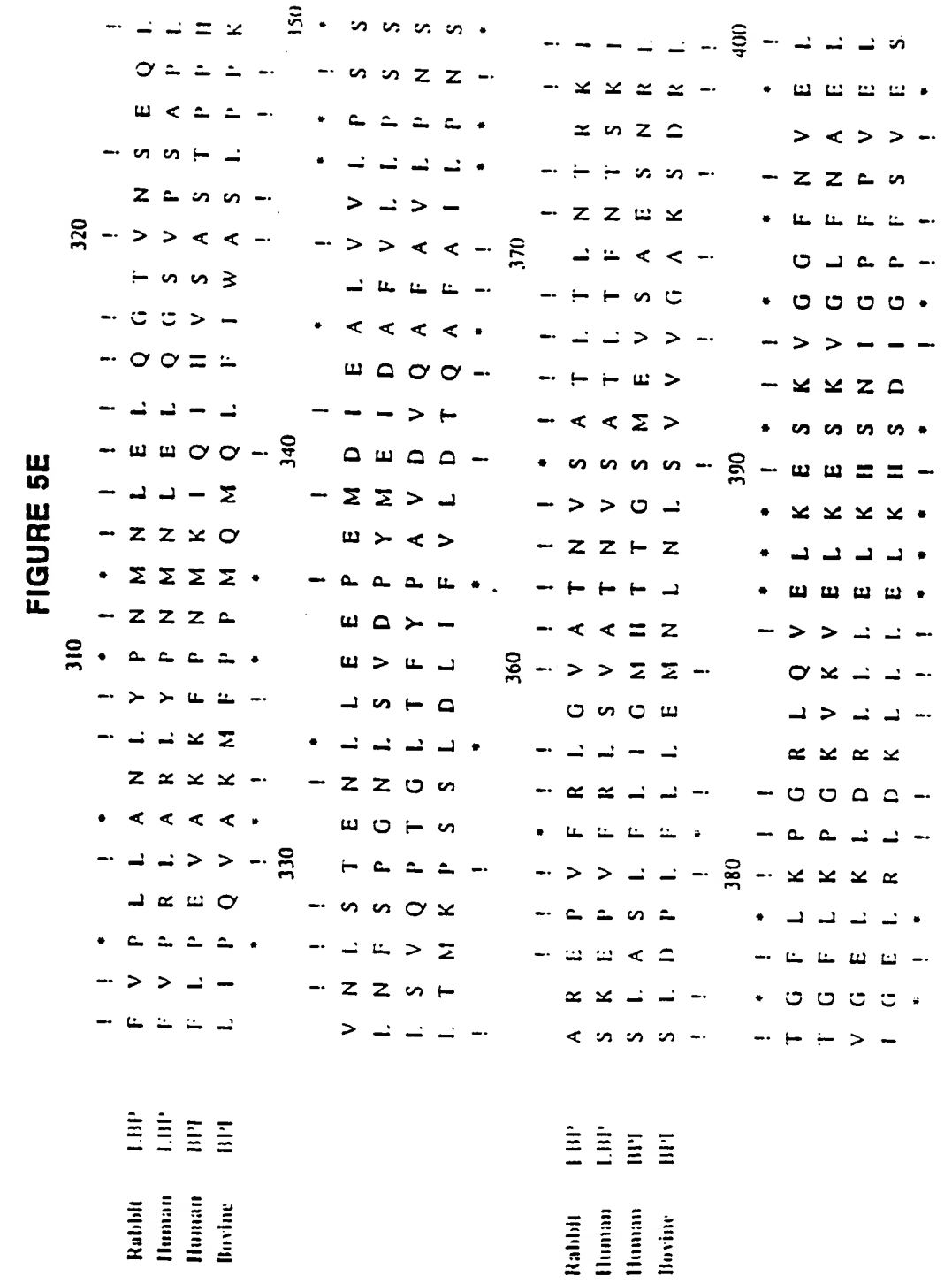

310
320
330
340
350
360
370
380
390
400
Rabbit IBP
Human IBP
Human BPI
Bovine BPI

FIGURE 5F

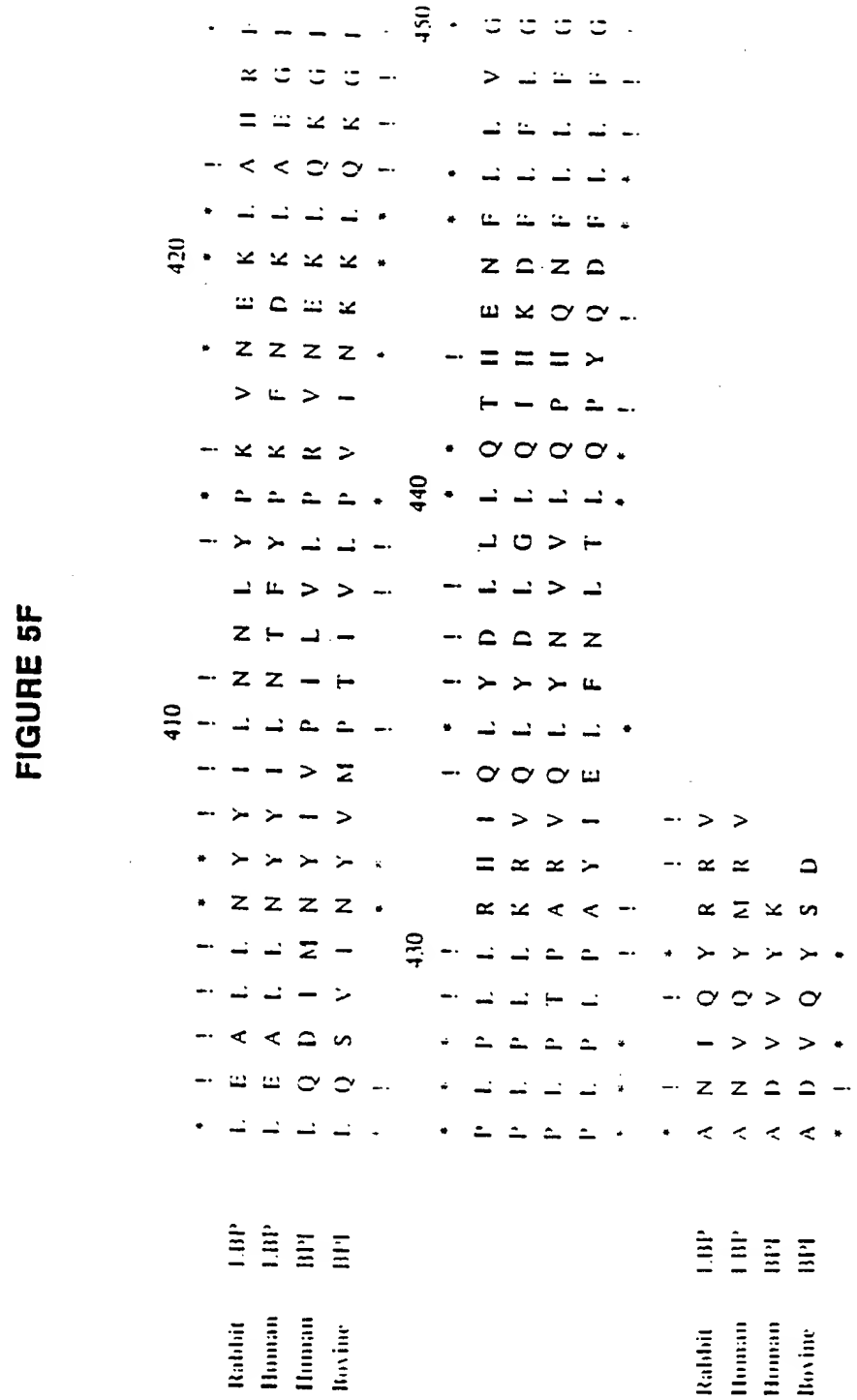


FIGURE 6

```
                10                  20                  30                  40                  50                  60
N- MGALARALPS ILLALLLTST PEALGANPGL VARITDKGLQ YAAQEGLLAL QSELLRITLP
                70                  80                  90                 100                 110                 120
   DFTGDLRIPH VGRGRYEFHS LNIHSCELLH SALRPVPGQG LSLSISDSSI RVQGRWKVRK
               130                 140                 150                 160                 170                 180
   SFFKLQGSFD VSVKGISISV NLLLGSESSG RPTVTASSCS SDIADVEVDM SGDLGWLLNL
               190                 200                 210                 220 25/30 230                 240
   FHNQIESKFQ KVLESRICEM IQKSVSSDLQ PYLQTLPVTT EIDSVAGINY GLVAPPATTA
               250                 260                 270                 280                 290                 300
   ETLDVQMKGE FYSENHHNPP PFAPPVMEFP AAHDRMVYLG LSDYFFNTAG LVYQEAGVLK
               310                 320                 330                 340                 350                 360
   MTLRDDMIPK ESKFRLTTKF FGTFLPEVAK KFPNMKIQIH VSASTPPHLS VQPTGLTFYP
               370                 380                 390                 400                 410                 420
   AVDVQALAVL PNSSLASLFL IGMHTTGSME VSAESNRLVG ELKLDRLLLE LKHSNIGPFP
               430                 440                 450                 460                 470         479
   VELLQDIMNY IVPILVLPRV NEKLQKGFPL PTPARVQLYN VVLQPHQNFL LFGADVVYK* -C
```

FIGURE 7

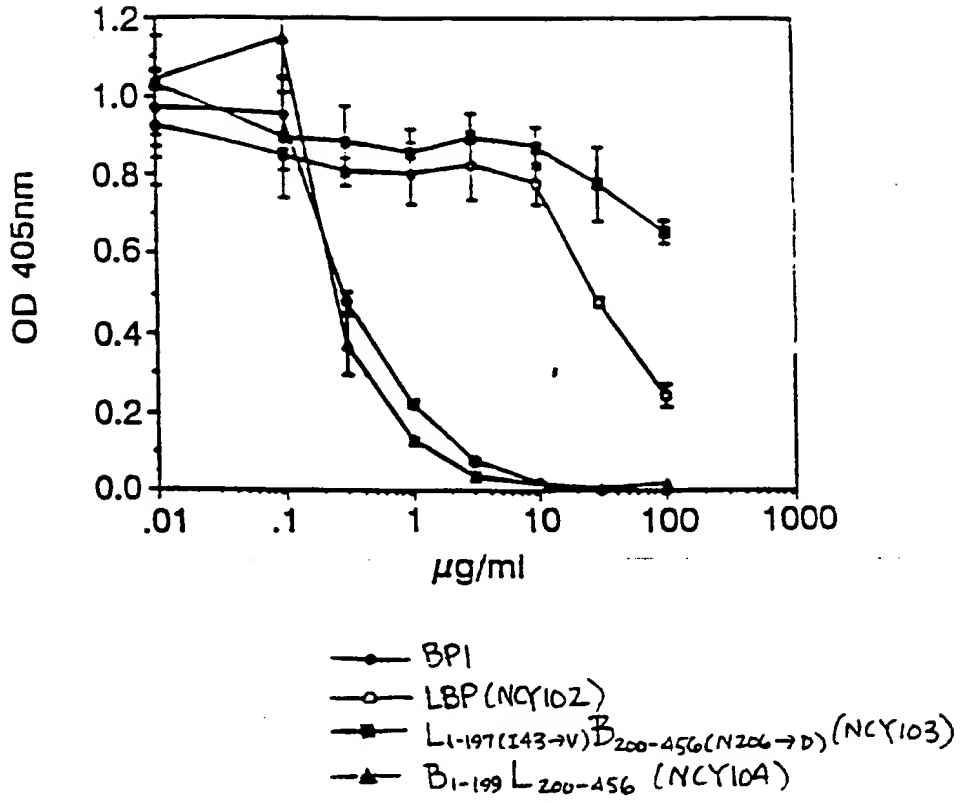

1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD 405nm
.01
.1
1
10
100
1000
µg/ml
BPI
LBP (NCY102)
L1-197(I43→V)B200-456(N206→D) (NCY103)
B1-199 L200-456 (NCY104)

FIGURE 8

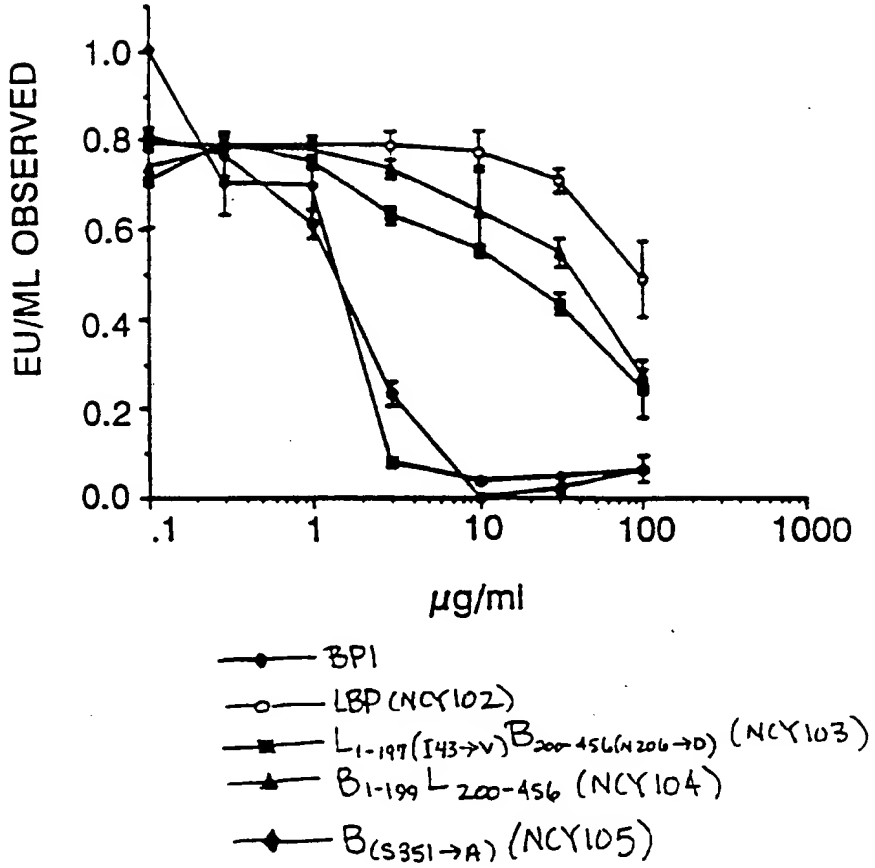

EU/ML OBSERVED
1.0
0.8
0.6
0.4
0.2
0.0
.1
1
10
100
1000
µg/ml
BPI
LBP (NCY102)
L1-197(I43→V)B200-456(N206→D) (NCY103)
B1-199 L200-456 (NCY104)
B(S351→A) (NCY105)

FIGURE 9

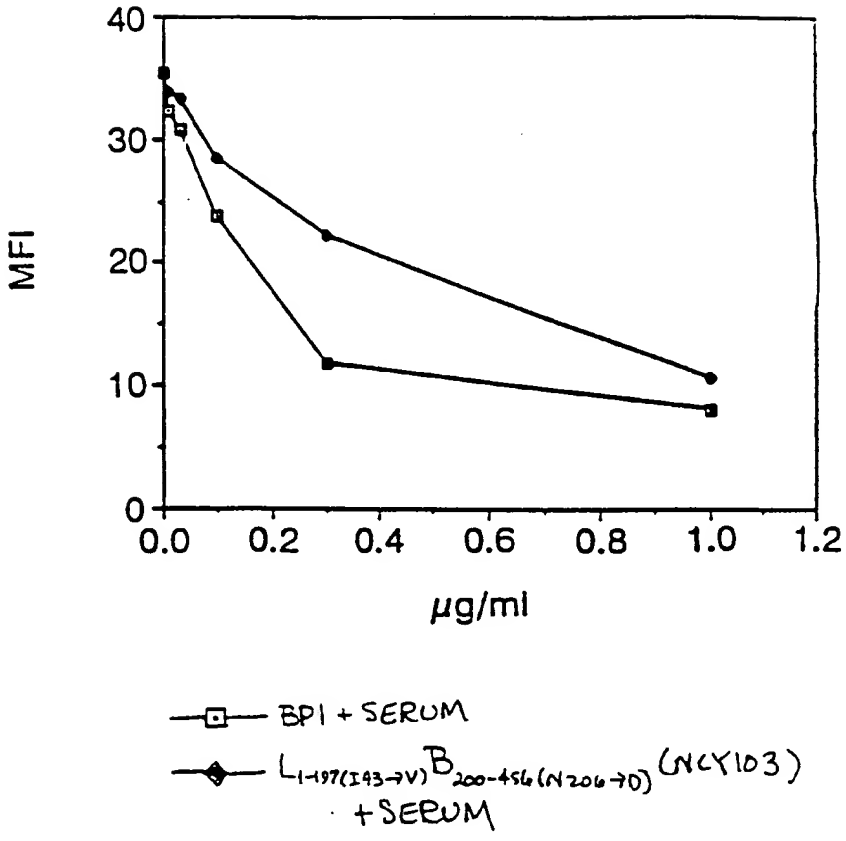

40
30
20
10
0
MFI
0.0
0.2
0.4
0.6
0.8
1.0
1.2
µg/ml
BPI + SERUM
L1-197(I93→V)B200-456(N206→D) (NCY103)
+SERUM

FIGURE 10

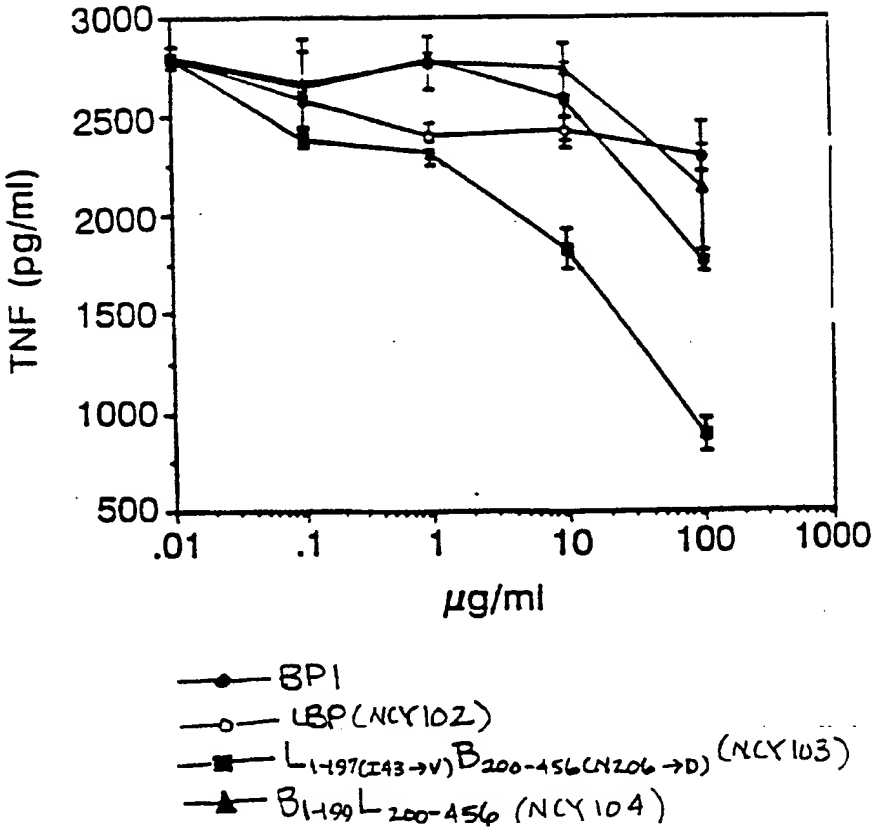

3000
2500
2000
1500
1000
500
TNF (pg/ml)
.01
.1
1
10
100
1000
µg/ml
BPI
LBP (NCY102)
L1-197(I43→V)B200-456(N206→D) (NCY103)
B1-199L200-456 (NCY104)

FIGURE 11

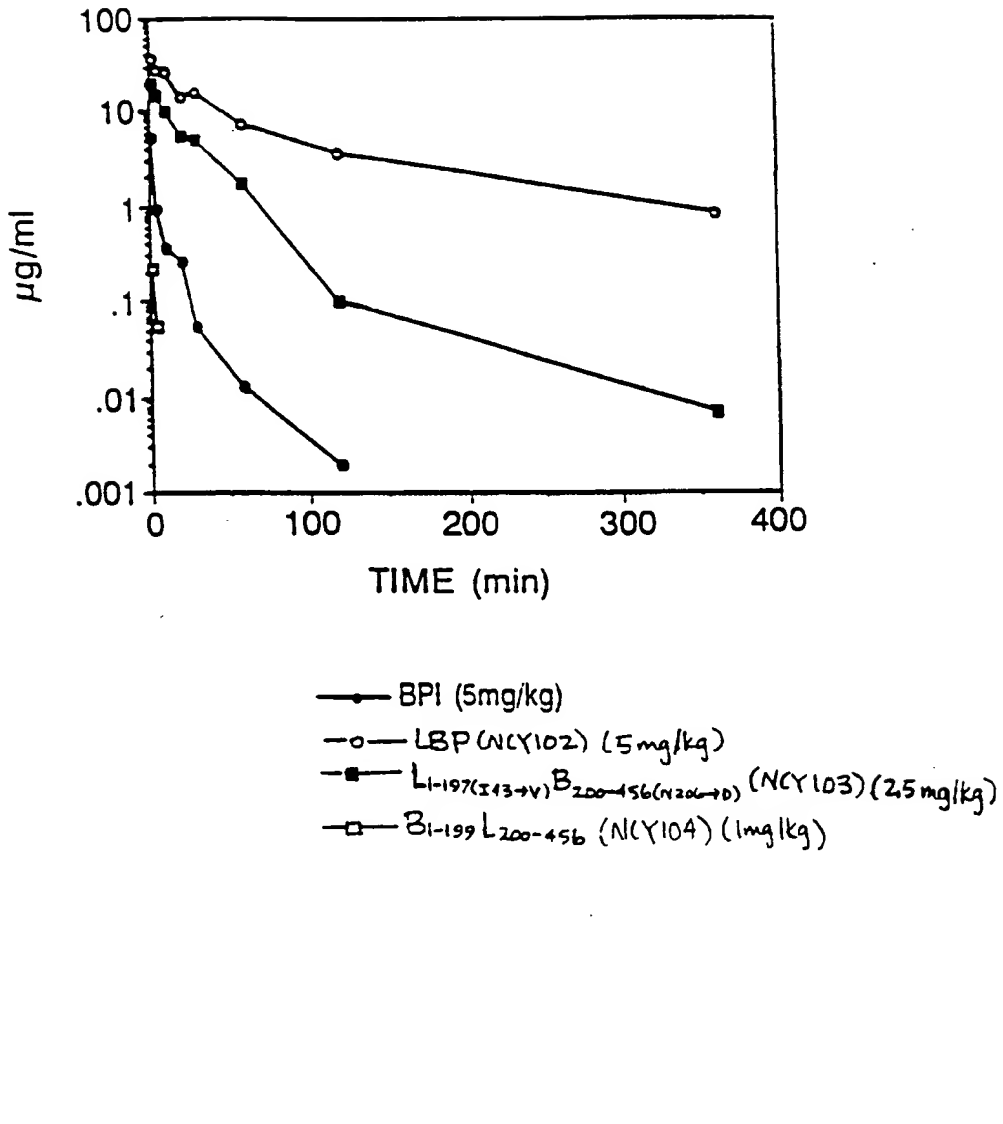

100
10
1
.1
.01
.001
µg/ml
0
100
200
300
400
TIME (min)
BPI (5mg/kg)
LBP (NCY102) (5mg/kg)
L1-197(I43→V)B200-456(N206→D) (NCY103) (2.5mg/kg)
B1-199 L200-456 (NCY104) (1mg/kg)

FIGURE 12

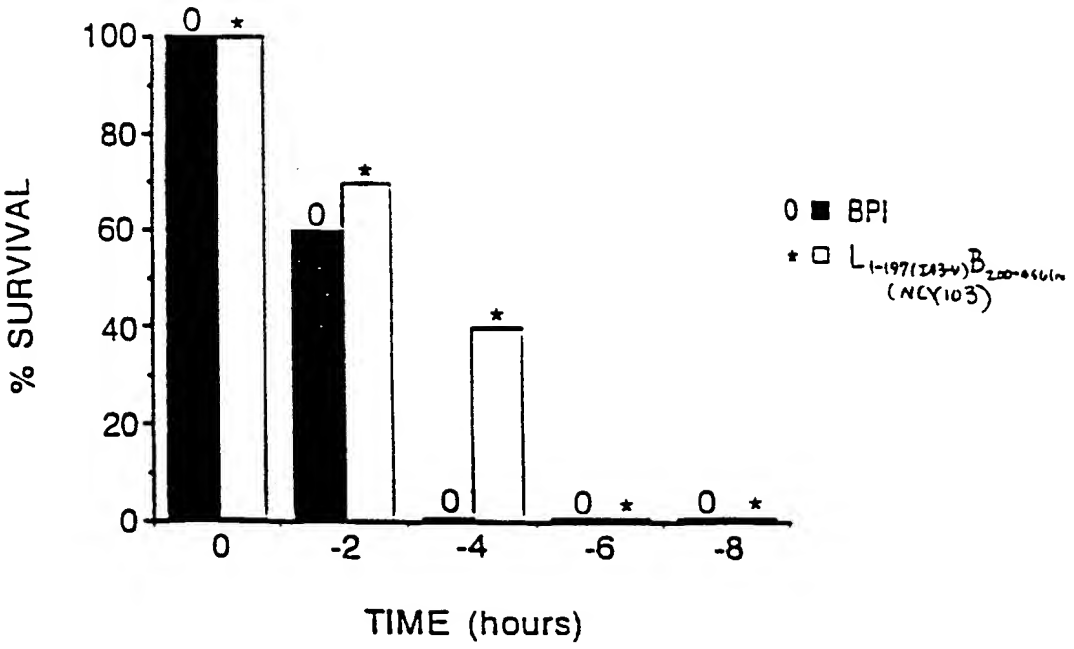

100
80
60
40
20
0
% SURVIVAL
0
-2
-4
-6
-8
TIME (hours)
0 ■ BPI
* □ L1-197(I43-V)B200-456(N)
(NCY103)

FIGURE 13A

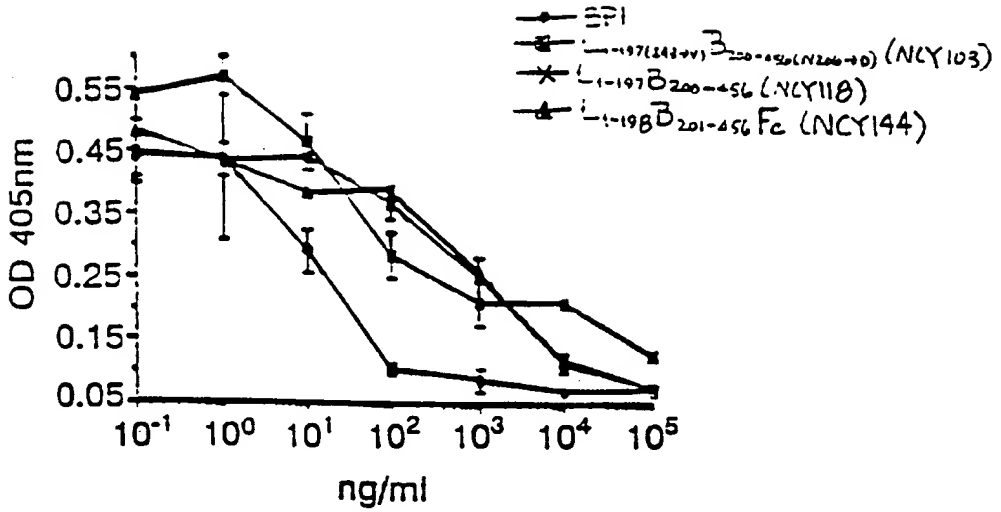


FIGURE 13B

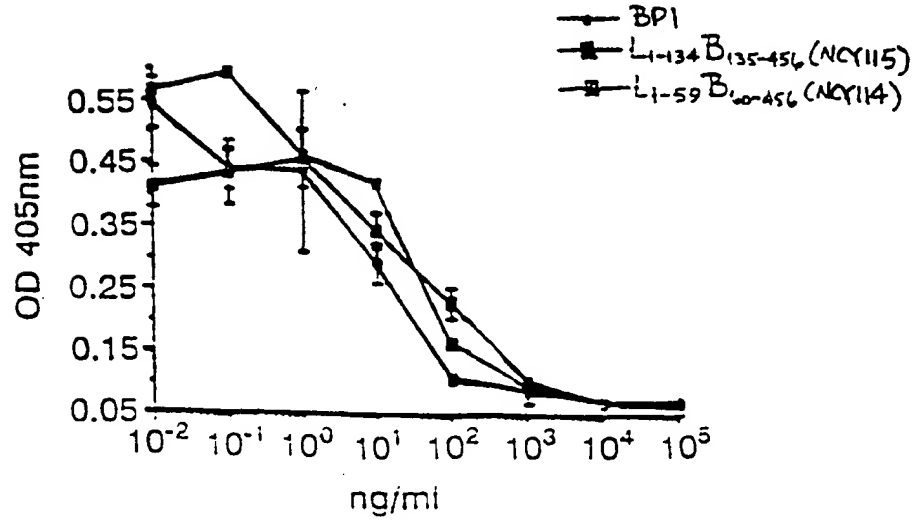


FIGURE 13C

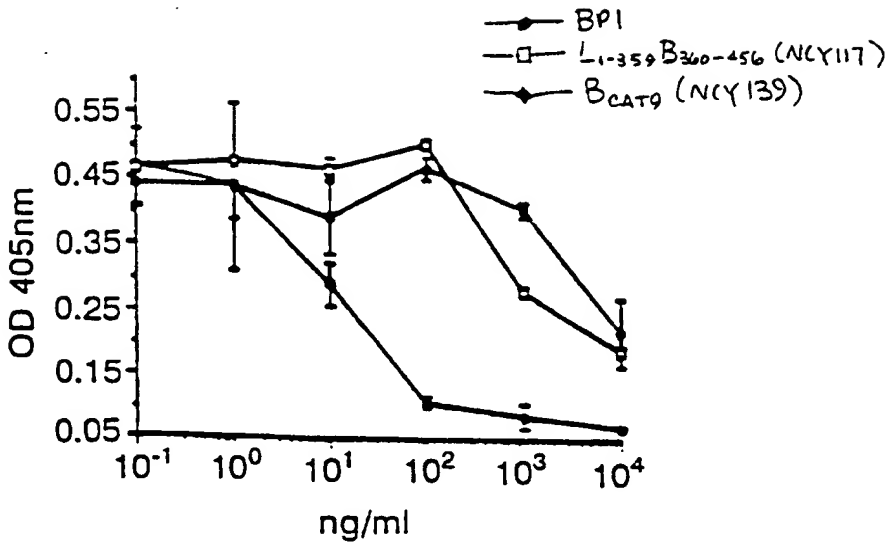

BPI
L1-359B360-456 (NCY117)
BCAT9 (NCY139)
OD 405nm
0.55
0.45
0.35
0.25
0.15
0.05
10-1
100
101
102
103
104
ng/ml

FIGURE 14A

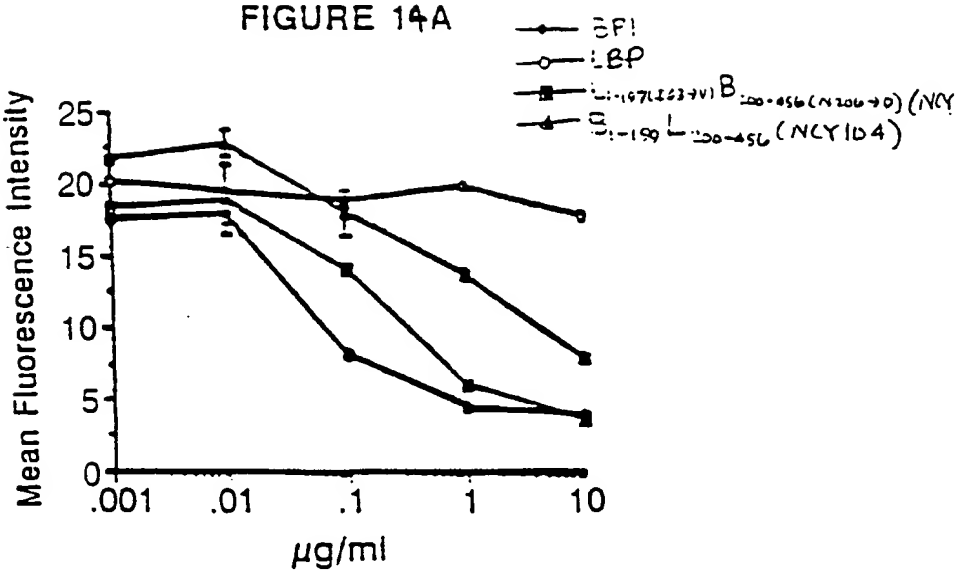


FIGURE 14B

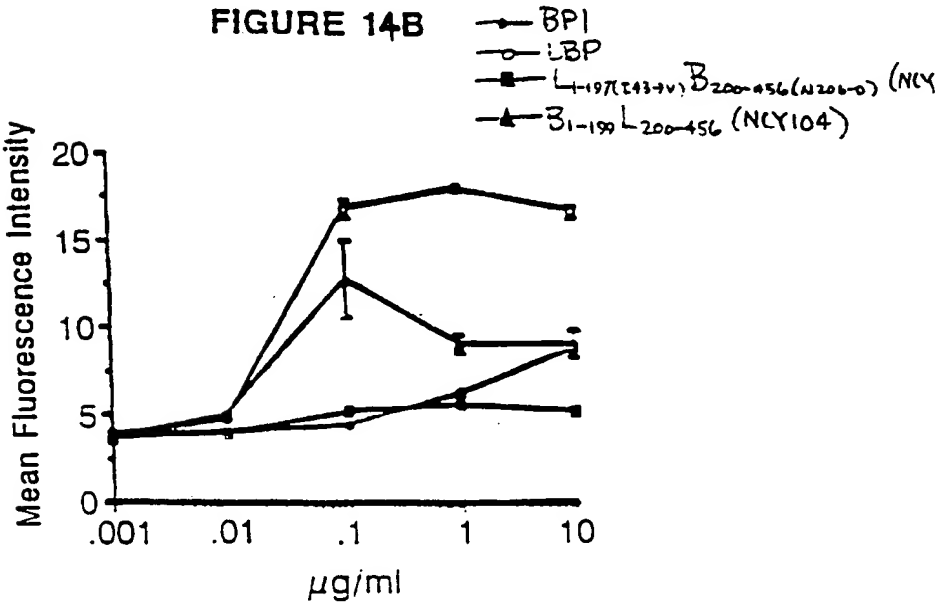


FIGURE 15

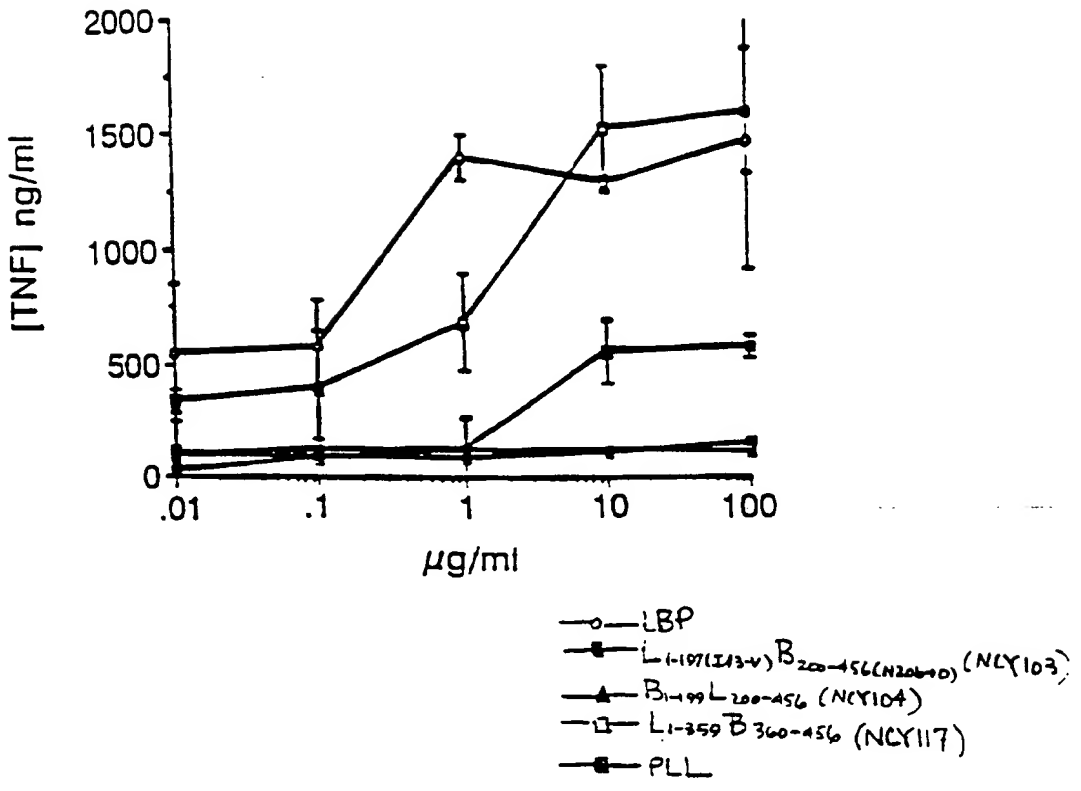

2000
1500
1000
500
0
[TNF] ng/ml
.01
.1
1
10
100
µg/ml
LBP
L(1-197(I13-V))B(200-456(N206-D)) (NCY103)
B(1-199)L(200-456) (NCY104)
L(1-359)B(360-456) (NCY117)
PLL

FIGURE 16

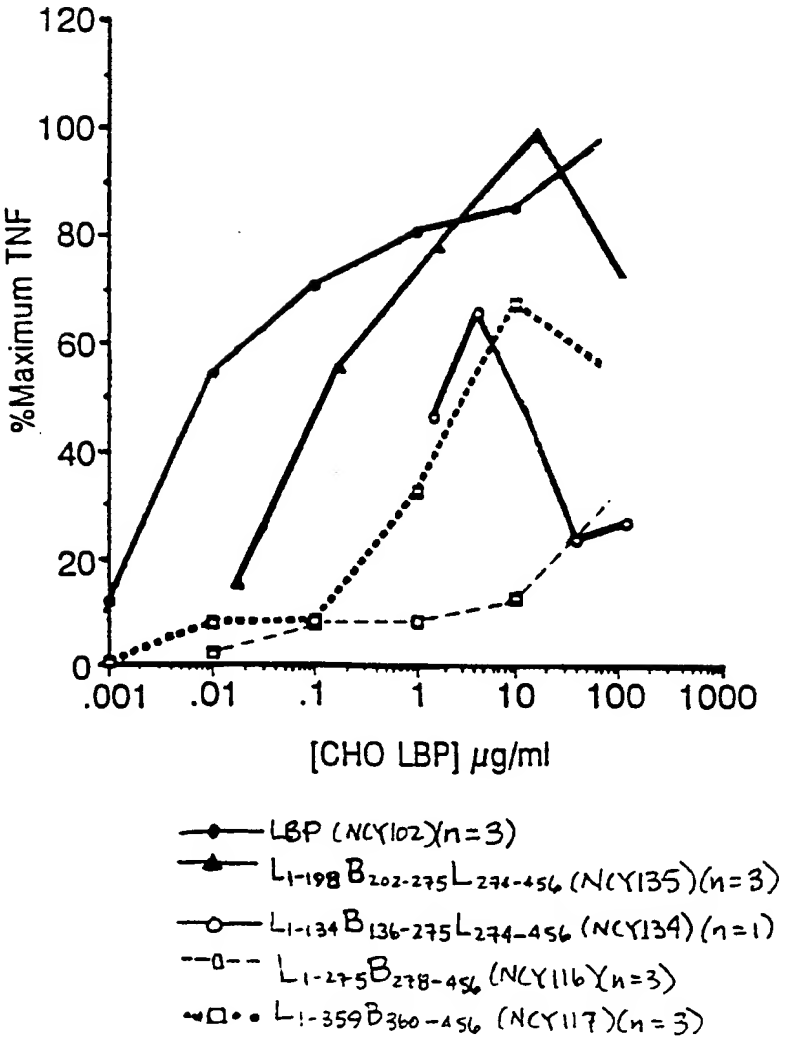

120
100
80
60
40
20
0
%Maximum TNF
.001
.01
.1
1
10
100
1000
[CHO LBP] µg/ml
LBP (NCY102)(n=3)
L1-198B202-275L274-456 (NCY135)(n=3)
L1-134B136-275L274-456 (NCY134)(n=1)
L1-275B278-456 (NCY116)(n=3)
L1-359B360-456 (NCY117)(n=3)

FIGURE 17A

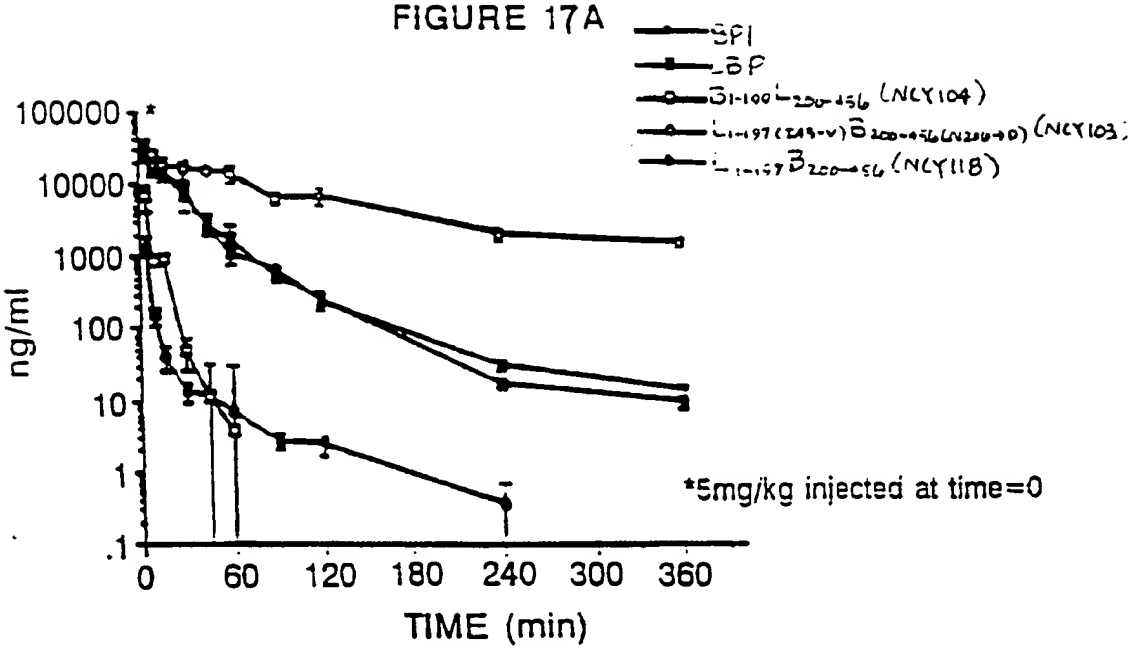


FIGURE 17B

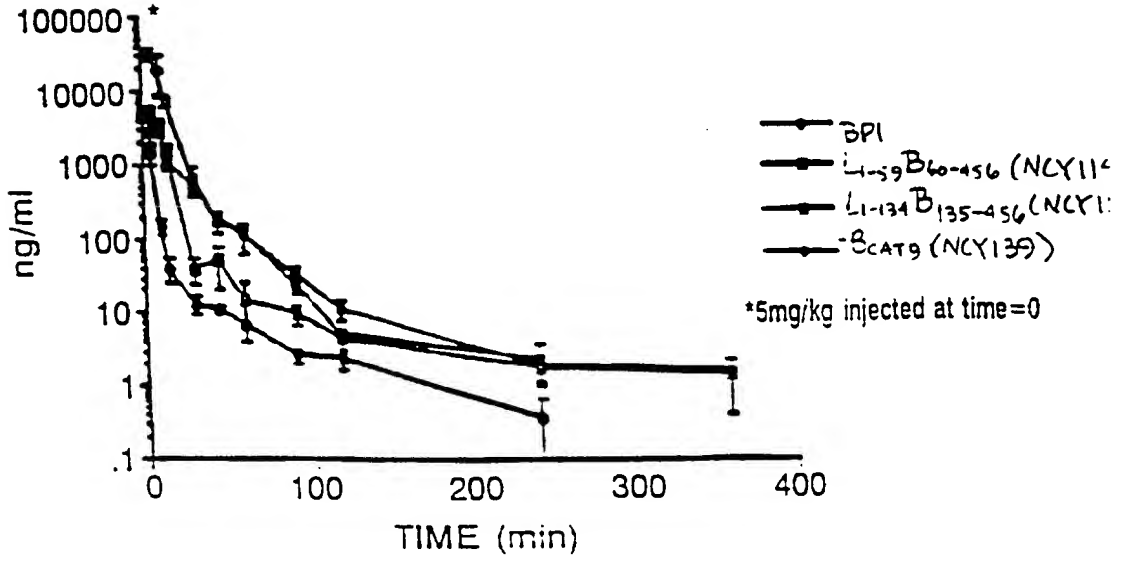


FIGURE 17C

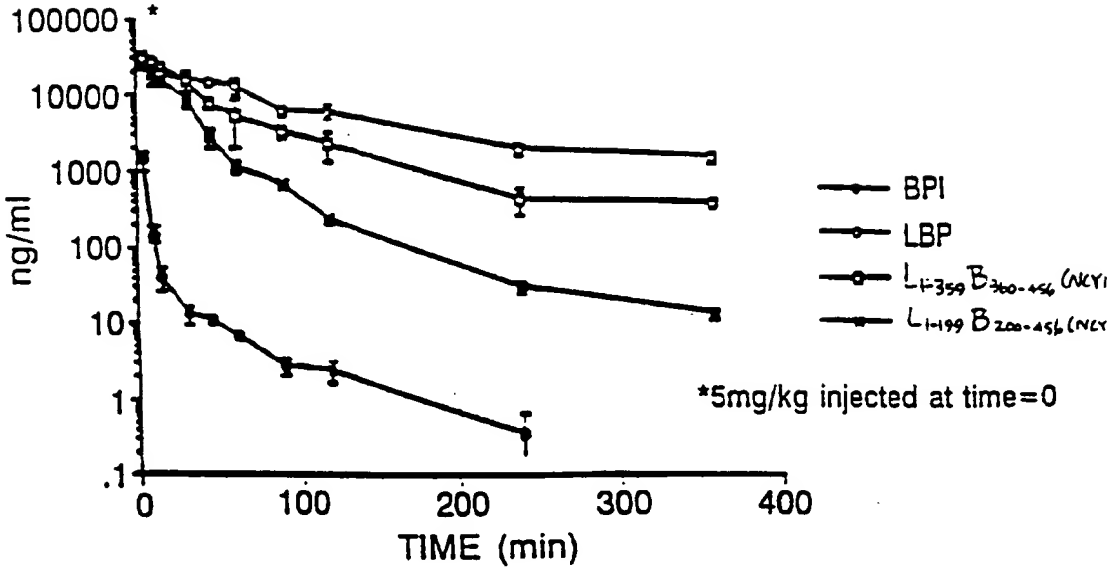


FIGURE 17D

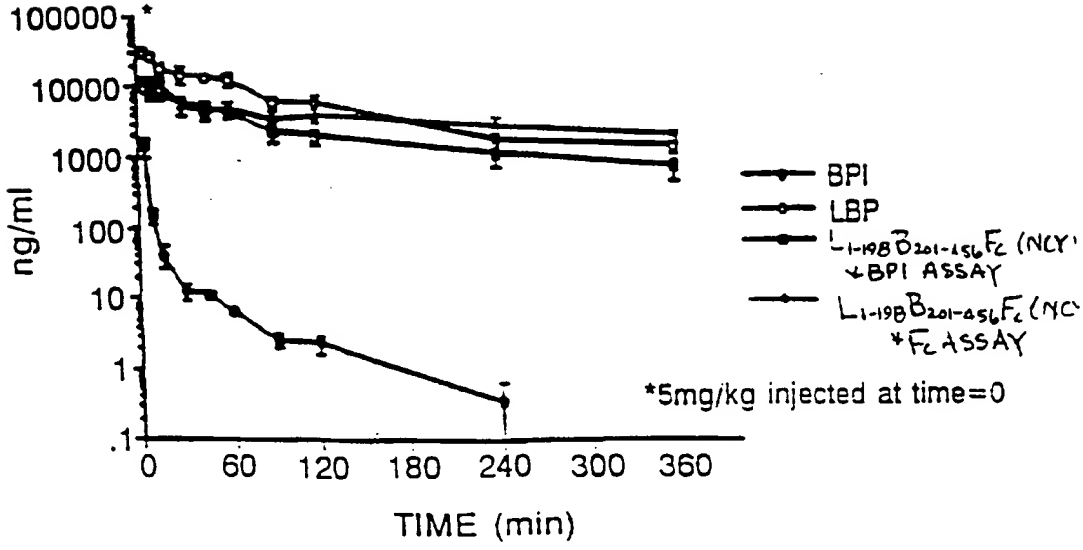


FIGURE 17E

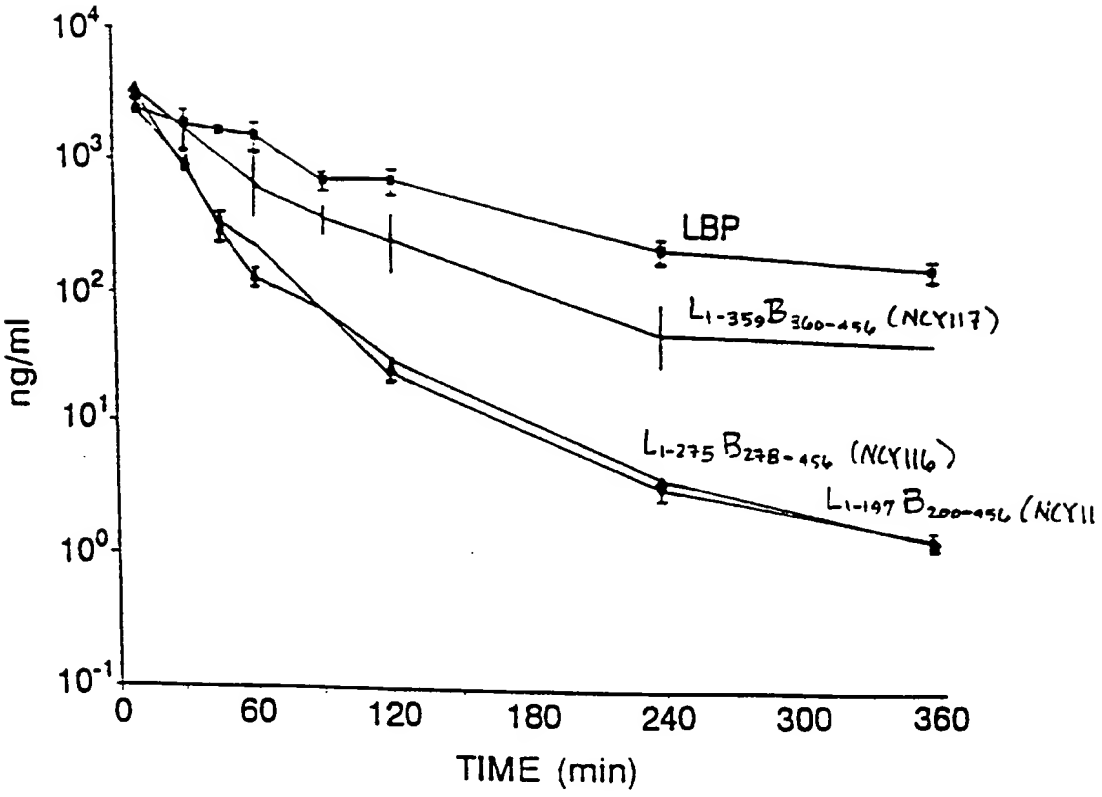

10^4
10^3
10^2
10^1
10^0
10^{-1}
ng/ml
0
60
120
180
240
300
360
TIME (min)
LBP
$L_{1-359}B_{360-456}$ (NCY117)
$L_{1-275}B_{278-456}$ (NCY116)
$L_{1-197}B_{200-456}$ (NCY11

FIGURE 17F

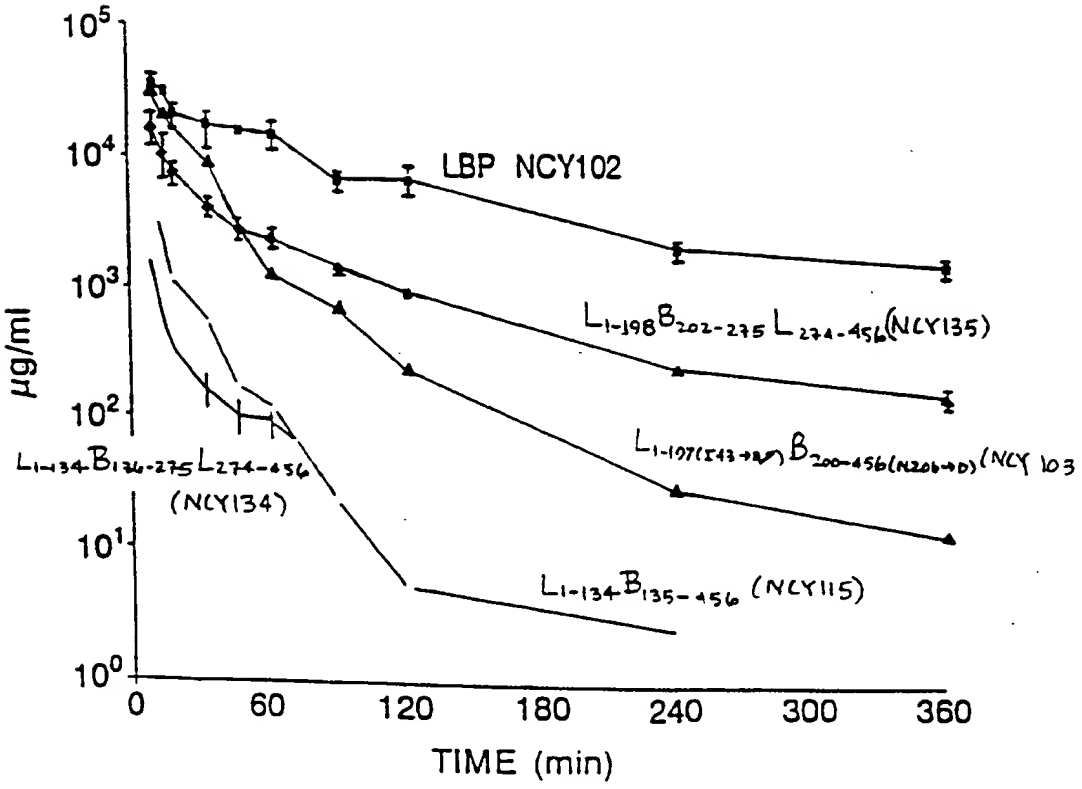
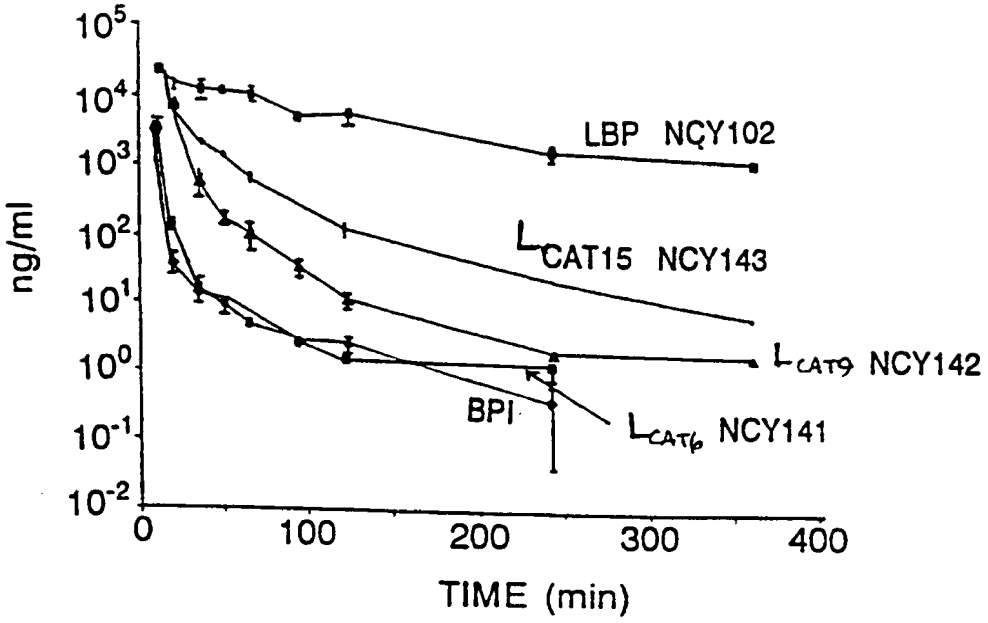

10^5
10^4
10^3
10^2
10^1
10^0
µg/ml
LBP NCY102
$L_{1-198}B_{202-275}L_{274-456}$(NCY135)
$L_{1-197(I43\to R)}B_{200-456(N206\to D)}$(NCY 103
$L_{1-134}B_{135-275}L_{274-456}$
(NCY134)
$L_{1-134}B_{135-456}$ (NCY115)
0
60
120
180
240
300
360
TIME (min)

FIGURE 17G



5mg/kg compound injected i.v. at t=0

FIGURE 17H

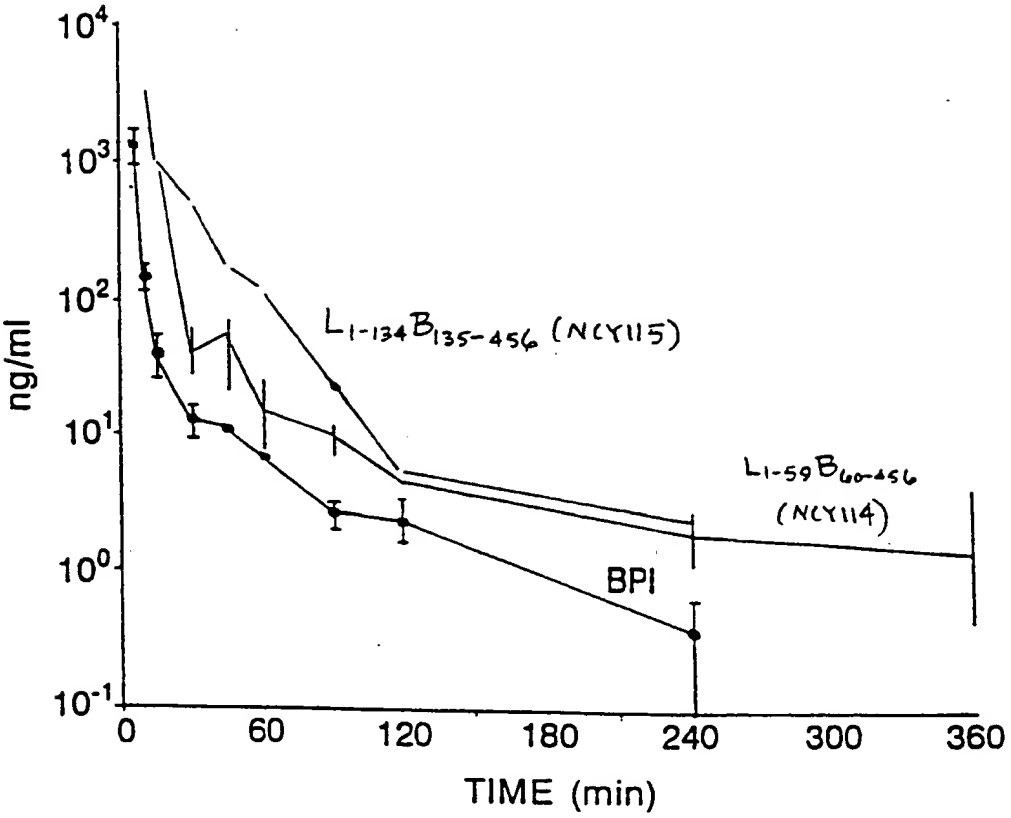

10^4
10^3
10^2
10^1
10^0
10^{-1}
ng/ml
$L_{1-134}B_{135-456}$ (NCY115)
$L_{1-59}B_{60-456}$
(NCY114)
BPI
0
60
120
180
240
300
360
TIME (min)

FIGURE 18

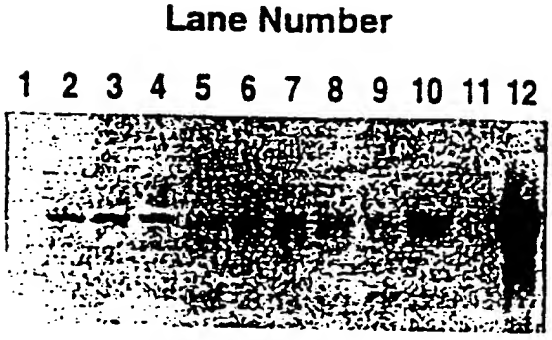


FIGURE 19

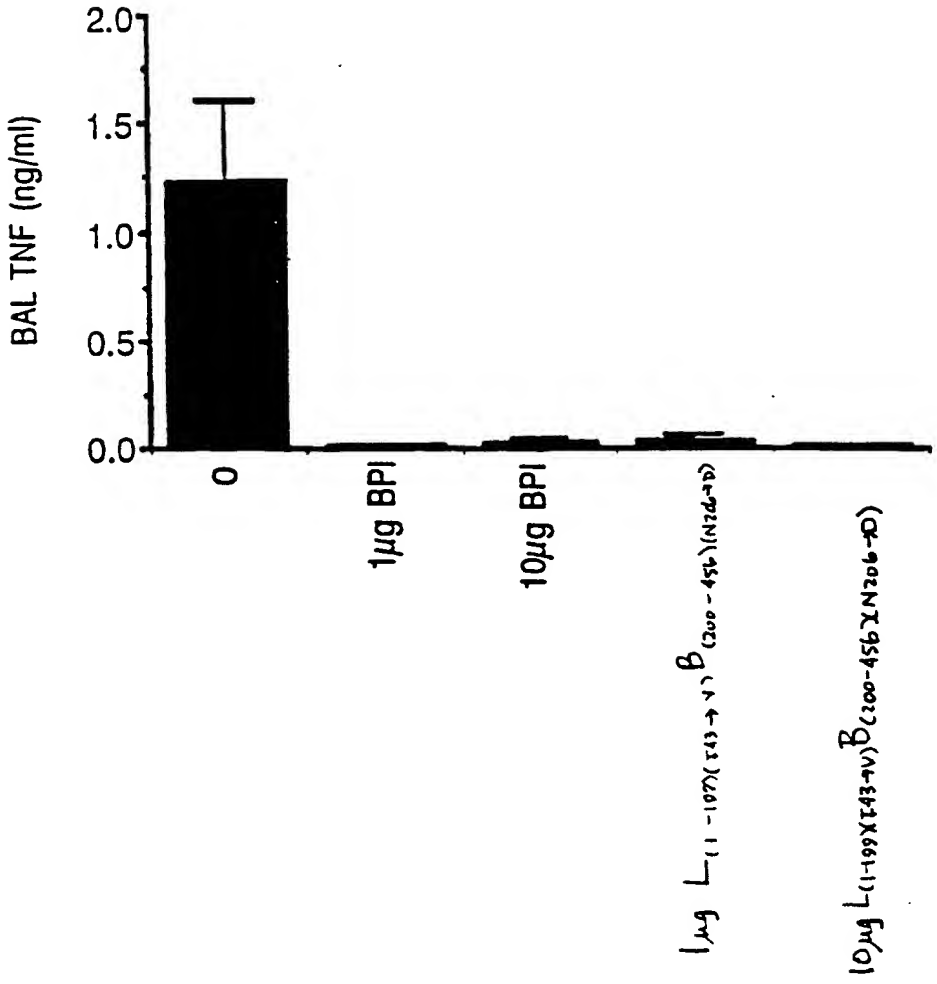
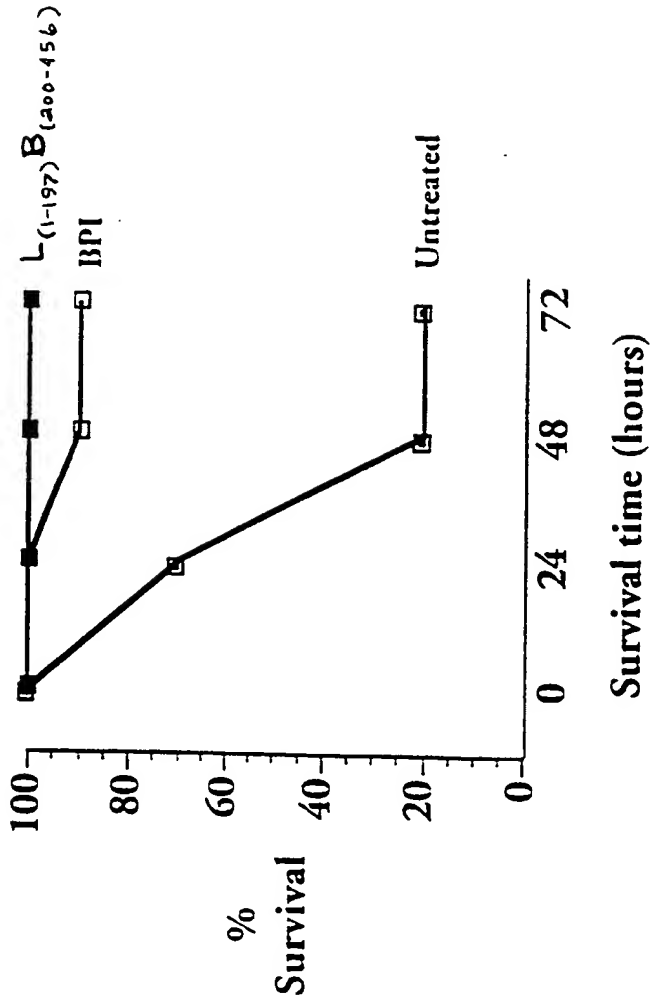

BAL TNF (ng/ml)
2.0
1.5
1.0
0.5
0.0
0
1μg BPI
10μg BPI
1μg L(1-197)(E43→V)B(200-456)(N204→D)
10μg L(1-199)(E43→V)B(200-456)(N204→D)

FIGURE 20


$L_{(1-197)}B_{(200-456)}$
BPI
Untreated
100
80
60
40
20
0
% Survival
0
24
48
72
Survival time (hours)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/06134

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 15/12; C12P 21/06; A61K 39/00, 38/00; C07K 1/00
US CL :530/300, 350; 435/69.1, 320.1, 252.3; 536/22.1, 22.3; 514/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/300, 350; 435/69.1, 320.1, 252.3; 536/22.1, 22.3; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,94/25476 A (SCOTT et al) 10 November 1994, see entire document.	1-38, 42
X	WO 94/18323 A (THEOFAN et al) 18 August 1994, see entire document.	14, 31, 32,
X	US 5,348,942 A (LITTLE et al) 20 September 1994, column 5, lines 40-45, column 7, lines 3-23.	14, 31, 32, 33

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 08 JUNE 1996	Date of mailing of the international search report 16 JUL 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer C Tsang for H. F. SIDBERRY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/06134

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
14-38, 42

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/06134

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-4, 9-13, 43, drawn to method of in vivo detection of a site of Gram-negative bacterial infection, labelled endotoxin neutralizing polypeptide, composition, classified in Classes 514, subclass 2.
Group II, claim(s) 5-8, drawn to a method of in vitro detection of a Gram-negative bacterial infection, classified in Class 436, subclass 517.
Group III, claim(s) 14-38, 42 drawn to endotoxin-neutralizing polypeptides, method of making the polypeptides classified in Classes 530 and 435, subclasses 300, 350; 69.1.
Group IV, claims 39-41, drawn to DNA encoding a recombinant endotoxin binding polypeptide, vector and host cells, classified in Classes 536 and 435, subclasses 22.1, 23.1, 23.5 and 320.1 and 69.1.
Group V, claims 44, 45, drawn to methods of treatment using an endotoxin neutralizing polypeptide, classified in Class 514, subclass 2.

The inventions listed as Groups I, II, III, IV, IV do not relate to a single inventive concept under PCT Rule 13.1 because,under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
Group I is directed to a labelled endotoxin neutralizing polypeptide which is not defined. The polypeptide may be a variant of BPI or LPS in that the amino acid must not be identical to the amino acid sequences of either BPI or LPS. The methods of Group I and II are in vitro or in vivo, the method steps will differ in parameter, reagents and method steps.
The inventions of Group III are directed to polypeptides which are different from that required in Groups I and II to preform the methods of detection. The polypeptide of Group III is not the special technical feature set forth in the inventions of Group I and II. Group IV is directed to DNA which encodes a an endotoxin neutralizing polypeptide. However, variants of BPI and LPS occur spontaneously or may be achieved by chemical modifications.
Group V is directed to other methods which use the endotoxin neutralizing polypeptide, but not one which is labeled as of Groups I and II.
The special technical feature which links the inventions is known in the art, for Groups I, II and III, as SCOTT et al WO 94-25476, discloses an endotoxin neutralizing polypeptide wherein the serine at 351 has been changed to alanine. (see page 18, lines 22-27. Other variants of BPI are disclosed by SCOTT et al at page 35.
This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

(a)species of BPI variants which are chimeric: claims 29-35
(b)species of BPI variants which have substitutions: 14-28, 36-38.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: the first species are directed to constructs comprising BPI or LPS and/or an immunoglobulin fragment. The second species is directed to BPI variants which have substitutions in the amino acid sequence. The variants have only substitutions. The chimeras are comprised of the LPS or the immunoglobulin fragment and thus are comprise an element not need in the variant BPI proteins.